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Review

of

INTRAMURAL RESEARCH

1965



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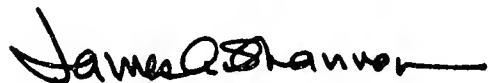
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FOREWORD

Here is the fifth review of Intramural Research from the National Institutes of Health. As the title implies the volume summarizes work done by the scientists in our own laboratories and clinics and in the offices connected with these. It does not deal with the many extramural activities of the National Institutes of Health that support much of the biomedical research throughout the United States and some efforts in other nations.

This review covers the year July 1964 through June 1965. There was no volume for the period in January 1963 through June 1964 when we changed from a calendar year to the present fiscal year basis. The philosophy behind the review has not changed. The words are those of the scientists, who followed very general guidelines. The reports received only minimal editing. Thus, we have tried to present for the reader as direct a view as possible into the workings of our large, diverse and very active scientific community as it pursues the specific research goals given by Congress to the individual Institutes, which together comprise the principal research arm of the United States Public Health Service. The Institutes strive to extend fundamental knowledge about the health problems of man and thus to provide a base for the broader statutory concerns of the whole Public Health Service, namely, "the causes, diagnosis, treatment, control and prevention of physical and mental diseases and impairments of man."



James A. Shannon, M.D.

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NATIONAL CANCER INSTITUTE

INTRODUCTION

The Clinical Director has prepared a brief summary of the specific activities in the clinical branches which he wishes to call attention to from the clinical research programs of the National Cancer Institute. The reports of the laboratory chiefs should be consulted for specific details. At this time attention should be called to the broad study of acute leukemia. This program has been given emphasis by a special appropriation of the Congress in the amount of \$10 million. This has permitted an expansion of the efforts to identify a specific virus in the etiology of human acute leukemia, to develop additional research support for the study of acute leukemia in man and in experimental animals and to provide a means for additional research in the clinical management of patients with acute leukemia.

Studies of acute leukemia range from studies in the chemistry of the virus, through the numerous leukemia virus models in the experimental animal, through the chemotherapy of experimental animal leukemia to problems in the management and therapy of patients with acute leukemia.

A specific accomplishment during the last year is the remarkable increase in remission rate of acute leukemia in adults. The remission rate now reaches the order of 60 percent as contrasted to 5-10 percent 1-2 years ago; this by virtue of combination chemotherapy. The beginnings of studies on reverse isolation also hold considerable promise in the study of the clinical management and particularly in the ability to eliminate infection as a cause of complicating factors.

One of the very interesting aspects of the special appropriation for acute leukemia has been the creation of a large coordinated effort with approximately eight program chairmen, each of whom has a committee for the programming of research in a specific area.

While this specific effort in acute leukemia can be easily identified, the National Cancer Institute intramural research programs do encompass a broad range of activities in basic and clinical research in cancer. These range from studies of the genetic factors effecting the incidence of tumors in the mouse through studies of tissue culture, and the chemistry of viruses to clinical studies of the chemotherapy of trophoblastic disease and the use of radical surgery for tumors of the head and neck and uterine cervix, and to studies of the biochemistry and physiology of man as altered by a malignant tumor. Again attention is called to the reports of the laboratory and branch chiefs for details of the specific research accomplishments.

LABORATORY OF BIOCHEMISTRY

Cytochemistry Section

Dr. D. Burk, Dr. M. Woods, and Mr. J. Hunter believe that the insulin:anti-insulin regulation of glucose phosphorylation in the hexokinase reaction plays a major role in control of metabolism in normal and malignant cells. Previous work had demonstrated that the presence of this mechanism in melanomas, mammary cancer, Ehrlich ascites tumor (weak control), human myeloid and lymphocytic leukemias, plasma cell tumor of mice, rabbit neutrophils and total bone marrow preparations, mouse peritoneal macrophages and spleen, tissue culture fibroblast cell lines and sarcomas derived therefrom, and mouse kidney and mouse brain but not liver. With liver, as in the case of brain, there have been numerous reports denying the direct action of insulin *in vitro*. The glycolytic actions of normal mouse and rat liver of a remarkable series of chemically-induced rat hepatomas (developed by Dr. Harold P. Morris) have been studied. Previous findings as to the importance of the DPN/

DPNH₂ ratio in the triosephosphate dehydrogenase reaction as a secondary rate-limiting step in glycolysis have been found to be applicable to liver. In all cases, detailed analyses showed that the glycolytic activity of the hepatomas exceeded that of the corresponding host liver, and also exceeded the average rate of livers from a series of tumor-free rats. Even the most slowly-growing and least anaplastic of the hepatomas glucolyzed more than the corresponding host livers. It was possible to show that in the liver-hepatoma spectra there is a direct correlation between the rate of glucolysis and tumor growth rate, and that these are related to the susceptibility of glucose utilization to anti-insulin regulation. As would be expected, the affinity for glucose was much higher (K_m much lower) in the tumors than in liver, and was proportional to the degree of anti-insulin restraint. In both liver and the hepatomas, *in vitro* stimulation of glucolysis by glucagon-free insulin was obtained at critical glucose concentrations. Further studies of endotoxin action on carbohydrate balance (blood, sugar, liver glycogen), *in vivo* and *in vitro*, continue to support previous conclusions that these toxins exert an insulin-like action on mammalian tissues.

Examination of the effect of anti-cancer agents and hormonal systems that inhibit cancer metabolism and growth have been centered on those compounds which influence the DPN/DPNH₂ ratio at the triosephosphate dehydrogenase (TPDH) locus and were extended to various tissues, including liver and the Morris series of rat hepatomas. Under *in vitro* conditions commonly employed in determining the effects of chemotherapeutic agents on cells, the TPDH locus in certain tissues, particularly liver and hepatomas, may become a rate limiting reaction masking the true glycolytic potential of the cells. The interaction between liver or hepatoma cells and acridine dyes plus visible light was investigated. As in Ehrlich ascites tumor cells, DPNH₂-induced inhibition of hepatic glycolysis could be reversed by excess (10^{-4} M) acriflavine in visible light with oxidation of DPNH₂. These new results obtained with liver and hepatomas should be helpful in understanding and maximizing the action of chemotherapeutic agents that involve loci of coen-

zyme I (DPN) function, and in more accurately evaluating the effects of agents which act on glycolysis.

Studies on the effects of light-sensitized dyes on the metabolism and growth of tumors indicate that white light and methylene blue, acting together, greatly increase the DPN requirement for proper metabolic function of ascites cancer cells. The increased requirement of DPN by cells in the presence of methylene blue and light can be satisfied in varying degrees by exogenous DPN, depending upon dye concentrations below 10^{-4} M. The increased requirement for DPN may be due to peroxide formation with concomitant coenzyme destruction, to increased competition between DPN and photosensitizing dye for available hydrogen, or both. Glycolysis seems to be more sensitive than respiration to the photodynamic effects of these dyes, and in this way, the photodynamic action is similar to the action of hydrogen peroxide and X-irradiation. Under proper conditions, photodynamic action of photosensitive dyes, like X-irradiation, may prove useful in cancer chemotherapy.

Biochemical studies on normal and leukemic leukocytes are being directed by Dr. W. H. Evans to determine the biochemical factors regulating the growth, maturation and phagocytic capacity of leukocytes, especially with regard to the arrested maturation and decreased phagocytic activity of leukocytes from myelogenous leukemia. It has become necessary to develop techniques for the rapid fractionation of cell types from bone marrow and blood so that the biochemical investigation of cell maturation can proceed. One of the major obstacles to successful cell fractionation has been overcome. This has been the tendency of dispersed marrow cells to reaggregate. Treatment of bone marrow cells with DNAase and trypsin followed by washing and resuspension of cells in a medium containing sodium acetate suppresses aggregate formation. A new sedimentation method is now being developed in collaboration with Dr. E. A. Peterson to resolve and separate these enzyme-treated bone marrow cells. Studies on the relative roles of catalase and myeloperoxidase in leukocyte peroxidative metabolism suggest that catalase serves as a peroxidative enzyme in intact cells

but only accounts for part of the total peroxidative metabolism. The extent to which myeloperoxidase contributes to the total peroxidative metabolism of intact leukocytes is not yet clear.

Nucleic Acids Section

In attempting to explain the accumulation of dCDP-choline and dCDP-ethanolamine in the Novikoff hepatoma, studies of the deoxyribose-containing compounds in normal, regenerating, and cancer tissues have been advanced on several fronts by Dr. W. Schneider and Dr. W. G. Fiscus (Research Associate). The preparation of labeled nucleotides has presented difficulties when chemical methods were used. However, this problem has now been solved by the use of enzymes present in rat liver supernatants. In the presence of ATP and phosphoglycerate, yields of 55 percent or more of H³ or C¹⁴-labeled CDP-choline, dCDP-choline, CDP-ethanolamine, and dCDP-ethanolamine were obtained in a two hour incubation with rat liver supernatant fractions. With rat liver supernatants as source of the phosphorylase, it was also possible to synthesize phosphocholine C¹⁴ and phosphoethanolamine-C¹⁴ from ATP-choline and ethanolamine in yields approaching 100 percent.

The two enzyme fractions that are involved in the synthesis of dCDP-ethanolamine and dCDP-choline can be differentiated by their isoelectric points, by their precipitability by salts, and by the fact that the former enzyme shows no activation phenomena. The latter enzyme, phosphocholine-cytidyl transferase, has been shown to be localized in the supernatant of rat liver and hepatoma homogenates and shows a remarkable increase in activity upon aging at 0° or 37°. The studies of this activation phenomena have indicated that the activation is induced by the phospholipid fraction of rat liver. A commercial preparation of egg lecithin which was obviously degraded was also highly active, but highly purified egg lecithin which had been protected from degradation showed little activating until it had been exposed to unfavorable conditions and even then was greatly inferior to the most active egg lecithin or rat liver phospholipid preparations. Studies of DNA synthesis in hepatoma have

suggested that the mechanism of incorporation of dCDP-ethanolamine into DNA was through dCTP rather than by direct incorporation.

The discovery that altered phospholipids activate the CDP-choline and dCDP-choline synthesizing enzymes is of fundamental interest and may be a most important finding. Although it is not yet clear what alteration is necessary in the phospholipid before it can serve as an activator it suggests that an unusual mechanism is operative in providing a constant supply of lecithin in the cell. Thus, it is visualized that as lecithin is broken down in the cell, the breakdown product or products activate the enzyme responsible for synthesis of the immediate precursor of lecithin, CDP- or dCDP-choline. This would provide a unique function for phospholipids and another method for maintaining cellular lecithin levels.

Several recent reports have claimed that DNA is present in Neurospora, rat liver and kidney, and calf heart mitochondria in amounts less than one percent of the total tissue DNA. In order to verify this finding and establish that contamination was not present, mitochondria were isolated from rat liver. The DNA fraction of the liver was isolated, freed of RNA by treatment with alkali, and hydrolyzed to the component bases. The nucleotide bases were separated by paper chromatography. This mitochondrial DNA showed a small but significant difference in the $\frac{A + T}{G + C}$

ratio as compared to nuclear DNA. Confirmation of the presence of DNA in mitochondria add to the growing body of evidence that these organelles contain their own DNA and thus may be self-duplicating and transmitters of hereditary information in their own right. Since tumors are known to be deficient in mitochondria, it suggests that the point of attack of a carcinogen or carcinogenic virus may be at the level of mitochondria.

The metabolism of the acid soluble deoxyribosyl compounds of animal tissue has been studied by Dr. J. Rotherham who has found that the total amount of deoxyribosyl compounds excreted in the urine of rats was lower when the animal was fed a synthetic diet containing no preformed source of these compounds than when animals were fed laboratory chow. At

least a part of these endogenously-formed compounds were probably not derived from *in vivo* degradation of deoxyribonucleic acid. The concentration of deoxyribosides in the interstitial fluid of two different tumors was lower than in the circulating fluid and the concentrations in the interstitial fluid in a third type of tumor and in normal subcutaneous tissue were the same as in normal blood. Studies have also been initiated comparing the structural properties and metabolism of the nucleic acids and nucleoproteins of cultured cells and of tumors derived therefrom.

Dr. R. Kielley's research on the cellular mechanism and the control of DNA synthesis has been concerned with the factors influencing the synthesis of thymidine triphosphate (TTP) in normal and tumor cells, i.e., the enzymatic conversion of thymidylate (TMP) to high-energy phosphorylated forms by the action of TMP-kinase. The particle-bound TMP-kinase previously found in the twice-washed mitochondrial fraction of mouse liver, has now been more precisely localized in the "large granule" fraction which corresponds more nearly to intact mitochondria free of lysosomal and microsomal contaminants, thus supplying confirmative evidence that intact mitochondria contain bound TMP-kinase and tends to discount the possibility of non-specific adsorption or enzyme concentration in some less well-defined particle in the crude mitochondrial fraction. In mouse liver, the enzyme causing degradation of TMP, the TMP-nucleotidase, was found in greatest amounts in the soluble fraction and not in the microsomal fraction. The ability to recover TMP-kinase from mitochondria of mouse liver probably is due to the weak mitochondrial nucleotidase activity. It has been shown that the TMP-kinase reaction in hepatoma extracts can be more rapidly limited by substrate depletion by addition of the soluble fraction of mouse liver which contains most of the TMP-phosphatase activity. Addition of other liver cell fractions caused little effect. The rate of a kinase reaction remained linear provided there remained excess TMP and provided ATP resynthesis was maintained with a high degree of efficiency. When ATP resynthesis was supported by glycolysis, complete recoveries of the combined kinase activities of mouse liver and

hepatoma could be obtained. On the other hand, when conditions of ATP resynthesis by oxidative phosphorylation were permitted, only 50 percent of the combined kinase activities could be obtained. These plus more complete kinetic data suggest that in addition to ATP concentration itself, which was fairly well maintained by oxidative phosphorylation even when kinase activity was markedly inhibited, the breakdown and dismutation products, ADP and AMP might act as inhibitors of kinase reaction. These results provide some evidence that the state of equilibrium of the adenylate system, $\text{ADP} \rightleftharpoons \text{AMP} + \text{ATP}$, may be an important factor in the regulation of DNA precursor synthesis in normal and tumor cells.

As part of a continuing study on the function and relationships of the structure and sequences of nucleic acids, work on the nucleotide distribution in nucleic acids, on the development of fractionation procedures, and on the preparation and characterization of nucleotide sequences has been continued by Drs. G. W. Rushizky and H. A. Sober. In collaboration with Dr. B. P. Doctor of the Walter Reed Army Medical Center, nucleotide and oligonucleotide composition and analysis of two well-separated serine transfer RNAs was accomplished. Fractionation of pancreatic RNase digests showed that the two components (serine II and serine III RNA) had identical base ratios and mono- to tetranucleotide composition. However, serine II RNA had twice as much ApUp than did serine III RNA. Conversely, the serine III RNA contained twice as much (AG)GU than did serine II RNA. The latter tetranucleotide was identified as being either AGGU or GAGU. While the nucleotide sequence analyses included only about 70 percent of the total RNA of each component, repetition of hydrolysis with another RNase may provide the remaining 30 percent of RNA components.

Continued improvement in fractionation procedures of larger oligonucleotides has been accomplished by the use of buffers containing 7 M urea at pH 2.7 and at pH 4. At the lower pH, fractionation occurs according to the net negative charge of the oligonucleotides, i.e., in RNase T₁ digests which terminate in G, on the A+C/U ratio. At pH 4, taking advantage of the difference in ionization of adenylic and cyt-

idylic acids; separations based on the number of A and C residues in oligonucleotides of like chain length can be accomplished. In this manner, tetra through decanucleotides have been examined in RNA T₁ digests of MS 2 RNA.

Oligonucleotides isolated and purified in this manner have been used as primary standards for the computer library in studies of computational spectral analyses that are being performed in collaboration with Dr. A. W. Pratt and Mrs. J. N. Toal (Laboratory of Physiology, NCI). The recognition, purification and characterization of nucleases with hydrolytic activity directed towards specific nucleotide linkages has turned to the isolation of a group of DNases that are free of RNase activity and non-specific phosphodiesterase activity. These enzymes have strict requirements for metals and hydrolyzed DNA as endonucleases. Kinetic experiments have verified that, in common with other known DNases, these new DNases can only hydrolyze one DNA chain at a given site in the double strand of helix. The specificity of the DNases isolated resembles that of pancreatic DNase since only mono- and dinucleotides remain at the end of digestion. A mixture of enzymes with closely-related metal requirements and pH optima results in 3' phosphates of mononucleosides and facilitates the determination of base ratios in DNA. No DNases with unique specificities have yet been found. Several RNases that were previously isolated and characterized as metal-requiring enzymes have been employed for the hydrolysis of RNA since addition of EDTA provides a very mild procedure for the reversible termination of enzymatic hydrolysis under conditions where phenol extraction would interfere. The use of these non-specific RNases also provides means for the preparation of 2',3'-cyclic terminal-ended oligonucleotides.

A simplified procedure has been devised for the large scale preparation of *E. coli* phage MS 2. From 300 liters of *E. coli*, up to 19 g of phage can be obtained. Previously used procedures involve a series of steps which gave lower yields and which were difficult to scale up.

In collaboration with Dr. C. W. Lees (Research Associate) methods are being sought

for the purification of the Moloney Virus (Dr. J. B. Moloney, Laboratory of Viral Oncology, NCI). It has been found that viral activity emerged in the excluded fraction of G-200 Sephadex columns and was adsorbed on DEAE-cellulose. The activity could be eluted by introducing sodium chloride in concentrations ranging from 0.03 M to 0.06 M. Preliminary experiments with aqueous polymer, liquid-liquid partition indicate that viral activity is concentrated in the lower phase of a 5.35 percent sodium dextrose sulfate—8.0 percent polyethylene glycol system.

Oligoribonucleotides from RNA and synthetic ribonucleotide polymers have been used to study the formation of polylysine-RNA complexes (in collaboration with Dr. A. Yaron, Visiting Scientist, and Dr. S. Schlossman, Research Associate). Digestion of the soluble complex results in the formation of a precipitate. This insoluble RNA-polylysine complex consists of a fragment of the original RNA chain and polylysine in a lysine:nucleotide ratio of 1. The complex can be dissociated and the protected RNA sequence recovered and analyzed. No base specificity has yet been found but the protected sequence bears a direct relationship in chain length to the chain length of the polylysine used for protection. Thus, treatment with polylysine, $\bar{n}=100$, results in a protected RNA sequence of $\bar{n}=99$; the use of oligolysine, $n=13$ resulted in RNA chain length of $n=16$.

Deoxyribonucleotides from partial and complete digests of calf thymus DNA have been used for specificity studies with new DNases. Other deoxynucleotides prepared from rat spleen have been used to check their function as stimulants of plasma cell formation. Preliminary results obtained with Dr. M. Feldman, (the Weizmann Institute of Science, Rehovoth, Israel) indicate that deoxyribonucleotides ($\bar{n} \geq 10$) were able to reactivate the immune response in thymectomized, X-ray irradiated adult mice.

Nutrition and Carcinogenesis Section

Intensive examination of the enzymatic and metabolic characteristics of "minimal deviation hepatomas" and tumors of different growth

rate has been continued by Dr. H. P. Morris with the collaboration of some 25 laboratories throughout this country and abroad. The generalizations reported last year still remain true "that no two minimal deviation tumors are alike, and that their enzyme, metabolic and physiologic patterns show striking individual differences. Furthermore, no single biological or biochemical abnormality has been uncovered which is common to all of the "minimal deviation hepatomas." The extensive examination of these tumors can be summarized as indicating that the minimal deviation hepatomas form a spectrum of neoplasms, each differing from the other in enzyme level and metabolic pattern.

The original minimal deviation tumor 5123 can no longer be considered a unique transplantable liver tumor. New groups of such transplantable hepatomas including several new highly-differentiated hepatomas have been developed and are now being studied biochemically and histologically. Variations in the chemical structures of the inducing carcinogens have produced tumors at different sites and with variable rates of growth. Three rapidly-growing solid transplantable hepatomas have been converted by Dr. S. Odashima (Visiting Scientist, Laboratory of Pathology, NCI) to the ascites form but similar conversions have not succeeded with 17 others. None of the slowly-growing tumors have been converted to the ascites form.

Hepatomas in rats maintained on high cholesterol diets continue to synthesize cholesterol, implying the loss of the normal feedback control mechanisms on cholesterol synthesis, but to a variable extent; the slowest-growing tumor synthesized the most cholesterol. Slow-growing hepatomas have zero or borderline levels of glucokinase which are not correlated with growth rate. The feedback inhibition of thymidine kinase was inhibited by TTP and dCTP in the slowly-growing hepatomas. The activity levels of three glycolytic enzymes, glucokinase, phospho-fructokinase, and pyruvic kinase were elevated in rapidly-growing tumors suggesting that these tumors are geared to straight glycolysis whereas some of the enzymatic activities of slowly-growing tumors deviate in the opposite direction. Several slowly-growing hepatomas readily oxidized

fatty acids suggesting that fatty acid oxidation may be the major source of energy for slowly-growing hepatomas that have slight capacity for glycolysis.

Studies on the effect of carcinogens on the induction of gastric tumors in rats have shown that the combined effect of two carcinogens, 2,7-FAA and 3-methylcholanthrene were additive. Gastric adenomas were induced in rats even though the injection of 2-FAA had occurred at a site remote from the stomach, implying that the carcinogen or its metabolite must have penetrated the stomach wall from the peritoneal cavity or from the blood stream. This observation suggests that an experimental test system is at hand in which the mechanism of gastric carcinoma can be studied.

Additional studies on transplantable hepatomas have been performed by Drs. T. T. Otani and H. P. Morris, in which the relative distribution of the two isozymes of glutamic-oxalic transaminase has been determined. The effect of extrinsic agents on their relative distribution has also been studied in attempts to correlate the isozyme patterns and the growth rates of several transplantable hepatic tumors. In five tumors examined the two isozymes of glutamic-oxalic transaminase had electrophoretic mobilities that were similar to each other and to those of the normal liver glutamic-oxalic transaminase. Studies of the K_m values of the anionic component of the two isozymes has shown that this component appears to be the more variable, one with the values of the anionic components tending to approach the K_m of the anionic component of the normal liver enzyme as the growth rate of the tumor increases.

Examination, by Dr. H. P. Morris, Dr. H. Dyer and Mrs. B. P. Wagner of the role of tryptophan and indole in the induction of bladder tumors in rats ingesting N-2-fluorenyl-acetamide (2-FAA) has shown that bladder tumors can be produced in Fischer strain rats without the addition of tryptophan. In a strain of genetically-jaundiced rats ingesting 0.025% 2-FAA as many liver tumors developed as in non-jaundiced animals of the same genetic background. Lower levels of 2-FAA are now being fed to determine whether an overwhelming exposure to the carcinogen had been used.

Biochemical studies in experimental cancer by Dr. H. Dyer and Mrs. K. Lueders have revolved around the carcinogenesis and metabolism of 2-FAA and 2,7-FAA. Studies with monkeys have shown that both of these carcinogens can be recovered quantitatively in the excreta of monkeys. Chromatographic studies of metabolites indicate that 2-FAA may not be carcinogenic for the monkey. Fecal excretion of great amounts of unmetabolized 2,7-FAA following oral rather than parenteral administration suggests that this very insoluble compound should be administered intraperitoneally for testing carcinogenicity.

With rats fed 2,7-FAA for six months, slight differences observed in the mucin content of the glandular stomach and gastric juice, decreased volumes of gastric juice, and a higher pH and lower free and total acid compared with control rats, may reflect early changes related to the occurrence of neoplasia of the gastric mucosa of rats fed the same diet for 10 to 12 months. 2-F-diAA-induced liver nodules in rats incorporated decreasing amounts of radioactivity from a single oral dose of 2-FAA-9-C¹⁴ with progressive development of nodules from early states of hyperplasia to the frank appearance of hepatomas. This observation may reflect the protein binding of carcinogens by liver and the lack of binding by hepatoma proteins. Greater glucuronyl transferase activity was found in homogenates and microsomal fractions of transplanted, slowly-growing, well-differentiated hepatomas than in normal or host livers, and less than in fast-growing hepatomas than in liver. Kinetic behavior and stability studies have demonstrated the similarity of enzyme activities in liver and hepatomas.

β -Hydroxy- α -amino acids and their N-acyl derivatives are being prepared by Dr. T. T. Otani. The free β -hydroxy- α -amino acids as well as their N-acyl derivatives are pretested for growth inhibitory action on microbial systems to study amino acid metabolism and to detect possible anti-tumor action. Compounds exhibiting positive action such as the N-acyl derivative of β -hydroxynorleucine are being tested on tumors and intact mammalian systems and on isolated mammalian cells in culture. The small amount of activities resulting

have indicated that resolution of racemic mixtures and separation of diastereoisomers of these β -hydroxyamino acids are required. The resolved diastereoisomers and their N-acyl derivatives are being prepared, with the hope that an increased activity over that shown by the racemic mixture will be obtained. This class of compounds offers a new group of tumor-inhibiting agents with a mode of action unlike those now in use in that they react within the cell to release an unnatural amino acid and the chloroacetic acid moiety.

Protein Chemistry Section

The development of new principles and the improvement of older procedures for the fractionation and characterization of macromolecules of larger structures of biochemical interest, e.g., ribosomes and whole cells, is of major importance and continues to accrue dividends by application to specific research problems.

Such development has resulted in the novel micropath sedimentation technique which is being used by Drs. E. A. Peterson and W. Evans for the separation of leukocytes and myelocytes from other leukocytes in guinea pig bone marrow and in serum. The early separations obtained have been shown to be due to the tendency of the mature granulocytes, myeloblasts, and red cell precursors to form aggregates, a tendency not shared to the same extent by red cells and myelocytes. Since such aggregation would be a major obstacle to the further fractionation of leukocytes by any method, considerable effort has been expended to minimize this tendency. A systematic study of the effective agents on cellular aggregation suggested that nucleoprotein adhering to the cells might be a factor. An effective method of pretreating the bone marrow cells with a mixture of trypsin and DNase was adapted from a procedure developed by Madden and Burk of this laboratory in 1960 for the preparation of viable single cell suspensions from solid tumors. Additional factors in the improvement of the cell separation were the use of gentler means of dispersing the marrow, final filtration of the cells through porous polyethylene membranes, the addition of sodium acetate to the medium and the omission of calcium and magnesium.

Nearly all the cells of such a preparation passed through a slowly-rotating horizontal column of small pore polyurethane foam whereas most of the leukocytes of the early preparations were strongly retarded by large pore foams.

Although some separation has been obtained, sufficient resolution has not yet been accomplished. A variety of material in shredded and beaded form have been tested. Of all the materials tested, so far, IRC-50 offered the most promise in that few cells were adsorbed or trapped. Its effectiveness in fractionating the cells by sedimentation will be explored in long columns. Adsorption of cells to the materials tested was not correlated with presence of charge or its sign. Further examination of particles and ion exchange resins for the micro-pore sedimentation technique will be continued. Preliminary experiments have shown that it is simple to coat a polyurethane foam with a polymer containing clusters of positive charges that can be readily controlled by reasonable changes in pH, thus limiting the changes in salt concentration that would be necessary for elution.

In an investigation of cytoplasmic nucleoproteins and their role in protein synthesis Drs. E. A. Peterson, E. Kuff and L. Kedes (Research Associate) have been isolating cytoplasmic-ribonucleoprotein particles and their protein and nucleic acid moieties by a combination of centrifugal and chromatographic techniques for use in metabolic experiments. A comparison of liver ribosomes and ribosomes isolated from rabbit reticulocytes subjected to the chromatographic procedures has shown that the reticulocyte ribosomes emerge as a single peak whereas the liver ribosomes yield two peaks in the sodium chloride gradient with a prominent final peak eluted by trisodium phosphate. The reticulocyte ribosomes showed an unchanged chromatographic pattern on rechromatography whereas the liver ribosomes shifted in position from the first to the second peak. When preparations of reticulocyte monosomes and pentasomes obtained by zonal ultracentrifugation were subjected to the chromatographic procedures, the monosomes and pentasomes emerged at different salt concentrations, and these fractions retained their original ultracentrifugal

characteristics. When the reticulocytes were incubated with the radioactive-labeled amino acids and then lysed and subjected to chromatography, the profile of the radioactive label coincided with that of the ribosomes and almost 100 percent of this radioactivity was sedimentable. Centrifugation in a sucrose density gradient revealed a high specific activity in the polysomes, indicating that the chromatographic procedures were mild enough to avoid disaggregation of the active polysome fractions.

A comparison of a reticulocyte ribosome isolated by centrifugation with those obtained by chromatography indicated that they were nearly identical in their ability to incorporate C¹⁴ leucine in a complete system of co-factors. The chromatographically isolated ribosomes, however, were free of endogenous co-factors whereas ribosomes prepared by centrifugation incorporated 60% as much label without added co-factors as with them. In an *in vitro* system containing the required co-factors, rat liver ribosomes (monosomes obtained by this chromatographic procedure) were able to incorporate radioactive labeled phenylalanine when stimulated by poly-U. This incorporation increased in a nearly linear manner with increase in poly-U concentration. At the maximum poly-U concentration tested (100 gamma per ml) the activity of ribosomes prepared chromatographically was nearly identical to that of ribosomes (polysomes) obtained by centrifugation through a sucrose layer. The latter ribosomes were not stimulated to increased activity by the addition of poly-U.

The fact that ribosomes prepared chromatographically have a lower intrinsic incorporating activity than those prepared by centrifugation, yet can be brought to the same high level of incorporating activity by the addition of co-factors makes such ribosomes particularly useful in the study of the co-factors and the mechanisms involved in amino acid incorporation.

It has been observed that nucleic acids that can be presumed to be single stranded, such as denatured DNA and the most rapidly-labeled RNA, are adsorbed to ECTHAM-cellulose very strongly and emerge in the final Na₃PO₄ eluate. Preliminary experiments indicate this fraction can be released from the column by increased

temperature, promising a very useful means for their isolation.

As part of a continuing study on the homogeneity and structure of proteins Dr. N. Cummings (Research Associate) and Dr. H. A. Sober have begun to study the phenomenon of cryo-precipitation. Cryoglobulins are a class of serum proteins found in various diseases, most notably multiple myeloma, which are characterized by precipitation in the cold, cryo-precipitation. This has important clinical aspects, producing symptomology and loss of biologic function often vital to the patient. The mechanism of precipitation of proteins as a function of temperature is important and fundamental.

Further studies of the metalloproteins of human serum, undertaken in collaboration with Dr. B. Vallee (Harvard University) have been concerned with the nature and possible role of magnesium as a protein-bound metal. Fractionation of human serum on DEAE-cellulose has indicated that magnesium is associated with proteins of gamma-globulin mobility. However, dialysis, electrophoresis, Sephadex and polyacrylamide gel fractionation point to a loose binding between the metal and the protein, i.e., the existence of a magnesium-protein complex rather than a metalloprotein *per se*. Recently a technique combining analytical and preparative ultracentrifugation developed in collaboration with Dr. E. Kuff has been used in an effort to study the distribution of magnesium and serum without introducing electrical or ionic forces that may have previously disturbed or displaced the original metal-protein equilibrium. Preliminary results indicate that about 70% of serum magnesium is unbound and that the remaining 30% is bound to the albumin fraction. The magnesium-albumin association is relatively weak since the magnesium can be easily displaced to other serum protein fractions.

The properties of poly- α -amino acids, model substances closely analogous to proteins, are being studied by Dr. H. A. Sober and Dr. A. Yaron (Visiting Scientist) in collaboration with Drs. A. Berger and E. Katchalski of the Weizmann Institute of Science, Rehovoth, Israel. Separation, isolation, and characterization of lysyl oligopeptides, which are of interest as histone analogs, was accomplished, and

one quarter to one gram quantities of the oligomers from the dimer to the decamer were obtained. The series has been extended to other members of the series in yields of approximately 30 mg each. These individual oligomers of lysine of known chain length and purity were used for the preparation of α ,N-DNP-and carbobenzoxy-derivatives for rotatory dispersion and spectral studies and to study the effect of chain length on development of color with ninhydrin. Potentiometric titrations on this series of compounds have shown the gradual decrease with increasing chain length of the apparent pK of the ϵ -amino group from 10.6 to 9.9. Optical rotatory dispersion studies with Dr. G. Fasman of Brandeis University have shown that at pH 12 conformation (helicity) begins to appear with the octamer and increases with increasing chain length. Organic solvents stabilize the helix form with oligomers of chain length 15 or more but have no effect on long chain length polylysines ($n=100$) and appear to destabilize the "helix" forms of the octa-, nona- and decalysines. The higher polylysines ($n=100$) aggregate reversibly and form precipitates at higher temperatures (40°), the rate of aggregation increasing with temperature. The lower members of the series on the other hand did not aggregate or associate. The different effects of organic solvents and temperature on the lower and higher members of the series suggests that while hydrogen bonds are probably the dominant stabilizing factor in the long chain molecules, hydrophobic side-chain interaction is a major factor in the short helical structures.

Interaction of polylysines with polynucleotides and nucleic acids has led to the formation of nuclease-resistant insoluble complexes from which oligonucleotides segments can be obtained. The length of the protected RNA segment is directly related to the length of the oligolysine allowed to interact. Oligolysines of known chain length have been used in inhibition studies of immunological phenomena to determine the size of the antibody site. In collaboration with Dr. M. Sela and Dr. R. Arnon of the Weizmann Institute, it has been shown that maximum inhibition is obtained with oligolysines of the same chain length as that of the immunizing antigen.

Mono-substituted haptens oligolysines (α ,N-DNP-oligo-L-lysines) prepared and used in immunological studies have provided a chemically-defined homogeneous series which ranges from non-antigenicity to full antigenicity. These purified, chemically-defined antigens, differing in size by increments of one lysyl residue have been used by Dr. S. Schlossman (Research Associate) in studies of the chemical basis of antigenicity and the basis of the heterogeneity of antibody. In Hartley Strain and Strain 2 guinea pigs it has been found that the octamer and larger haptens oligopeptides are antigenic; smaller peptides are not antigenic. The L-configuration of the α ,N-DNP-haptens were essential for antigenicity. Both delayed and immediate sensitivity reactions, which were easily separated in these two strains of guinea pigs, had the same structural requirements and were both obtained from the same antigen.

The extracellular RNase from *B. subtilis* is being used by Dr. R. W. Hartley, Jr. and Dr. C. W. Lees (Research Associate) in their study of the relation between the structure of an enzyme, its function, and its synthesis. The small size, stability, absence of disulfide bridges, and easily measured activity of this enzyme, plus the fact that the genetic apparatus of this bacterium has been widely studied and manipulated, have suggested this ribonuclease as an excellent choice for such a study.

Nearly half of a gram of purified enzyme has been produced. The current product gives a single peak in gel filtration chromatography and only very faint bands of impurities can be seen by disc electrophoresis at pH 4.3 and at pH 2. Precipitating antibodies to the enzymes have been obtained and have shown that two bands appear in Ouchterlony double diffusion. Both antigens are found in the enzyme band in the pH 4.3 disc electrophoresis run. However, preliminary evidence suggests that only one of the bands possesses RNase activity.

Study of the heat and pH stability characteristics of the enzyme has temporarily founded due to the relatively large amounts which can be irreversibly adsorbed to glass and plastic surfaces. The rate and perhaps the amount of such losses depend on the salt concentration, pH, and temperature and on the specific cations

present. All bivalent cations greatly reduce the rate of loss. Iron, however, affords protection which exceeds all others by several orders of magnitude. In the presence of 10^{-4} M FeCl_2 no detectable loss on glass surfaces can be found at room temperature after 48 hours.

RNase from an independently isolated *B. subtilis* strain has been partially purified and shown to resemble that of the original strain very closely, including immunochemical cross reaction. A number of mutants have been obtained that are deficient in the production of extracellular amylase and protease. One double mutant, producing neither of these enzymes, produces normal amounts of the ribonuclease. A report from another laboratory that a powerful inhibitor of the enzyme may be obtained from the cells of the same *B. subtilis* strains that produce the enzyme has been confirmed and efforts to isolate the inhibitor are under way.

The cytochemical organization of normal and malignant cells is being examined by Drs. E. Kuff and W. Hymer and Mr. N. Roberts, with special reference to the problems of the duplication of intracellular components. Transplantable plasma cell tumors in mice are presently the main objects of study since they provide a system for investigating relationships between a differentiated function (secretory protein synthesis) and neoplastic cell growth. Attention has been focused on the isolation and characterization of cytoplasmic RNA from the RPC-20 solid tumors.

The selective loss of a quantitatively small but metabolically active type of RNA when conventional phenol extraction procedures were used, led to the development of a novel method which permits direct sucrose gradient analysis of a total microsomal RNA without selective loss of actively-labelled components. The procedure is rapid. It is carried out at zero degrees and has been equally applicable to both RPC-20 tumor and liver, with yields of consistently between 87 to 100%. Furthermore, in this procedure de-proteinization with phenol is achieved with no loss in specific activity.

By means of this technique, rapidly-labelled RNA was obtained from both RPC-20 tumors and livers of tumor-bearing animals that sedimented as a sharp 18S peak superimposed

upon a heterogeneous size distribution ranging from 10S to more than 30S. The label did not appear in the sedimentation range commonly ascribed to mammalian, pulse-labelled "messenger RNA" between 4S and 18S, and it was only by exposure of the preparations to temperatures of 37–60° in low anionic strength that a displacement of the rapidly-sedimented, post-labelled RNA into the so-called "messenger" region of the gradients was obtained.

With Dr. C. Abel of Field Studies, preliminary study of the ability of RNA obtained in this manner to stimulate an amino acid incorporation in the cell-free *E. coli* system of Nirenberg has shown that "messenger activity" of the tumor RNA was at least partially preserved during the preparative procedure.

A subline of the RPC-20 ascites plasma cell tumor appears to have lost its capacity for synthesis of the characteristic RPC protein. This non-producing line has been examined by electron microscopy by Dr. R. Ziegel (Viral Oncology) and still retains the cytological appearances of a plasma cell tumor. The growth of this line has greatly increased over that of the remaining protein-producing RPC-20 lines. Dr. Ziegel has found occasional C-type "viral" particles budding from the surface of the non-producing cell, a phenomenon not previously associated with the murine plasma cell tumors.

Following intraperitoneal injection of ascites plasma cell tumors, the malignant cells tend to implant on the mesenteric surfaces and form solid nodules (in addition to the free cell population). When the mesentaries were examined with a dissecting microscope at varying times after transplantation of free ascites tumor cells, discrete nodules were discernible after 7 days post-transplant but not at 5 days. Individual nodules were easily dissectable, and when transferred to antigen wells of Ouchterlony plates and lysed with detergents, such nodules invariably liberated specific secretory protein in easily detectable amounts. Nodules from the non-producing ascites tumor lines gave no reaction with specific antisera. In the absence of detergent, nodules from the producing RPC-20 line gave no reaction with the specific antisera, indicating that the specific protein responsible for precipitant arcs was contained within the cells and did not repre-

sent contamination with ambient secretory protein.

Tumor-Host Relations Section

The interaction between the tumor and the host organism is being investigated at several different levels of organization ranging from the whole animal down to the molecular level and makes use of a variety of biological systems.

Dr. P. Gullino, by the use of a "tissue isolated" tumor preparation into which he has implanted a chamber for obtaining interstitial fluid, has studied the environment of the neoplastic cell and has found anomalous behavior in the tumor interstitial fluid as compared with other fluids of the host. As expected, administration of NaHCO₃ produced alkylosis in the blood lymph and subcutaneous interstitial fluid of the host, but acidosis in tumor interstitial fluid. The pH of this fluid normally about 0.3 unit lower than that of the host fluids, became 0.6 of a unit lower as a result of the bicarbonate treatment; the difference in bicarbonate increased from 3 to 5 mM per liter and the difference in CO₂ tension increased from 35 to 68 mm Hg. These remarkable modifications could be obtained with a relatively mild treatment, that is the substitution of drinking water by 0.1% of sodium bicarbonate solution. The difference of 0.3 of a pH unit and 35 mm Hg in CO₂ tension between the tumor and host interstitial fluid was also maintained during acidosis produced by ammonium chloride. Thus, an induced acidosis of pH 6.6 to pH 6.7 could be maintained in neoplastic tissue, a pH which is known to impair the survival of neoplastic cells *in vitro*. Inhalation of air containing 5 to 10% CO₂ produced an increase of the CO₂ tension of the tumor interstitial fluid to 200 mm Hg, about 100 mm Hg higher than that found in other body fluids of the host. These studies indicate that it is possible to produce a selective effect in the interstitial fluid of a tumor and should have implications in the design of chemotherapeutic procedures.

Since the tissue-isolated preparation allows sampling of both the afferent and efferent blood through the tumor as well as a measurement of blood flow, it has been possible to

study the degree of correlation between respiration and glycolysis of neoplastic tissues *in vivo* and *in vitro* and to evaluate whether the data collected from *in vitro* studies can be applied to neoplastic cell populations growing *in vivo*. Preliminary results indicate that the coefficient of oxygen utilization by the tumors studied range from 47 to 69% and the ability of neoplastic tissues to remove oxygen from the blood is equal to or higher than that of normal organs. Reduction of oxygen supply is partially compensated for by the tumor with an increase of blood flow but not by enhancing the oxygen-utilizing capacity. The overall consumption of oxygen by neoplastic tissue *in vivo* is influenced by a variety of factors and is difficult to assess. In the series of rat tumors examined, the values ranged from 0.5 to 8.0 mM/100g/hr. The higher values indicate that the Q_{O_2} *in vivo* may be equal to the Q_{O_2} that have been measured *in vitro* with surviving slices. Moderate hyperglycemia (200 mg/100 ml plasma) enhanced the oxygen consumption of the tumor but high levels of glycemia reduced the oxygen utilization, implying an *in vivo* demonstration of the Crabtree effect.

At the cellular level Dr. R. E. Greenfield is studying single cell suspensions from the tissues of normal and tumor-bearing animals. It has been shown that the respiratory rate of parenchymal liver cell suspensions at a pH of 7.0–7.2 is inversely related both to the concentration of cellular glycogen and to the glucose concentration of the media. Since cells isolated in the livers of male animals have, in general, higher levels of cellular glycogen than those from the female, cells from the male had lower respiratory rates than those from the female. At a pH of 7.6–7.8, on the other hand, respiratory rates had no relationship to either glycogen or glucose in the media. The respiratory rates of cells from the female at this pH were high, and indeed doubled those from male cells. Under culture conditions, cells from a regenerating liver still showed mitotic activity after three days and again after the fifth and sixth day.

In collaboration with Dr. S. Perry (Medicine Branch, NCI) Dr. R. E. Greenfield has been studying the fractionation of cell suspensions by gradient centrifugation. In cooperation with

Mr. G. Judson (Guest Worker) a new centrifuge of 50 ml capacity has been developed with a disc-shaped, batch-type head, and without vanes. The general distribution of the cells within the centrifuge head can be observed during the acceleration and deceleration. The centrifuge was found to be suitable for gradients for cells up to a density of 1.13; at higher densities viscosity introduced difficulties. Clumping was found to be one of the principal obstacles to the clean separation of cells but clumping could be decreased by the use of polyvinylpyrrolidone which had been dialyzed for 24 hours or passed through Sephadex G-50 to remove the smaller molecules. Red cells and cells from chronic myelogenous leukemia have been fractionated in gradients of polyvinylpyrrolidone both by differential and buoyant density centrifugation. Red cells were found to have a buoyant density range of 1.08–1.13. Lymphocytes, myelocytes, and polymorphonuclear leukocytes from chronic myelogenous leukemia cells had average densities of 1.03, 1.04, and 1.055 respectively.

Drs. R. I. Mishell (Post-doctorate Fellow) and R. E. Greenfield have been studying immune and malignant plasma cells in tissue culture, with the aim of establishing an *in vitro* system which will enable normal mouse spleen cells to be immunologically-induced to differentiate, proliferate, and function and which will permit malignant plasma cells to grow and synthesize myeloma globulins. The general procedure has been to modify culture conditions, assessing the response as measured by a quantitative assay which determines the number of cells in the population which synthesize specific antibody. The assay used is a modification of the agar hemolytic plaque technique developed by Jerne. Studies of spleens removed from mice at progressively longer intervals following a single *in vivo* immunization indicate that very few plaques (antibody synthesizing cells) can be recognized until 72 hours after immunization. A peak response is observed at 4 to 5 days after which the number of plaques formed declines rapidly. As a result of a number of modifications in culture conditions, it has been found that the oxygen concentration appears to be quite critical since oxy-

gen in excess of 20% is toxic to the development of plaque forming cells, but does not adversely affect plasma cells that have already differentiated. Low levels of oxygen (2%) is also inhibitory but by a clearly different mechanism in that more cells actually differentiate in 2 percent than in 20 percent oxygen but they do not survive as well. Furthermore, the toxicity of low oxygen is observed with previously differentiated cells as well as newly differentiating cells. The size of inoculum is also of extreme importance since inocula of 5×10^7 cells per cc are quite effective whereas very little differentiation can be detected with 1×10^7 or with 2×10^8 under the culture conditions employed. Fetal bovine serum seems to supply a factor or factors which are essential for differentiation in that 30% serum results in a 5-fold increase in the number of cells that differentiate as compared to 5% serum.

Dr. E. Shelton has just returned from a year of study in the laboratories of Dr. W. Bernhard, Laboratoire de Microscopie Electronique, Institut de Recherches sur le Cancer, Villejuif, France and has established an electron microscopy unit in the Laboratory of Biochemistry. During the year at Villejuif, a study was made of the effect of various fixing and embedding procedures on nucleohistone preparations. Of greatest interest were the results obtained when formalin-fixed material was embedded in 2-hydroxyethylmethacrylate (GMA). GMA-embedded material showed no structure under the electron microscope other than a uniformly dense sheet. Upon treatment with pepsin or tris buffer however a network of thick filaments appeared. Tris buffer is known to extract histones and although the protease pepsin does not act extensively on basic proteins, it appears to be quite effective on thin sections of formalin-fixed nucleohistone preparations. Similarly, when thin sections were treated only with DNase, filaments were again brought into evidence. When both pepsin and DNase were employed sequentially, all evidence of structure disappeared. These experiments suggest that conventional fixation and embedding procedures result in the loss of materials that are preserved by embedding in GMA. Selective enzyme digestion of GMA-embedded materials

reveals structures similar to those seen in the conventional preparations.

Diffusion chamber studies on factors effecting the malignant transformation of tissues have been continued. Collaborative studies with Dr. B. Arneson of Boston failed to confirm the report of Levey and of Miller in which immunologic competence of neonatally thymectomized animals was restored by implantation of diffusion chambers containing newborn thymus.

The establishment of two tumor lines of the same origin differing markedly in catalase activity without other apparent differences has provided an excellent system for the study of properties and interrelationships of cell populations by Dr. M. Rechcigl, Jr. and Dr. R. E. Greenfield. These tumors have remained stable for over 70 generations of transplantation. When fragments of tumor from the high catalase line and the low catalase line were mixed together and injected subcutaneously, stable tumors of intermediate catalase values have been obtained for up to 30 generations. However, on injection of fragments of the mixed tumor into the spleen, three-fourths of the metastases obtained in the liver had catalase values of 20-40 units whereas one-fourth of the metastases had catalase values of 90-140 units. Injection of the low catalase or high catalase tumor into the spleen gave only nodules with low (40 units) or high (200 units) levels of catalase, respectively. Whether the metastases from the mixed tumors with 90-140 units of catalase are still due to small mixed groups of cells or whether they are due to hybrid cells is not known. Of great interest is the observation that when small clumps of cells were prepared by the use of trypsin, PVP, and sucrose and were injected in different proportions of low and high catalase lines subcutaneously, the initial tumors had catalase activity values approximating the calculated mean catalase level of the mixture. However, within the next two generations the catalase line except that in the third generation that mixture which consisted of 99:1 of low catalase line to high catalase line produced a tumor characteristic of the low catalase line.

The physiologic role of catalase and related enzymes and the mechanisms of their regulation in normal and neoplastic tissues has been a

continuing line of investigation by Dr. M. Rechcigl, Jr. Using procedures previously described which are capable of separating catalase synthesis and catalase destruction, the depression of catalase activity observed in rats following the feeding of cycad meal or its toxic component, cycasin has been examined. It was found that whereas the rates of destruction were approximately the same in normal and cycasin-fed rats, the rate of catalase synthesis is roughly 20 percent of that in the normal rats. The results are comparable to the earlier findings in this laboratory where the lower catalase activity observed during protein deficiency was entirely explained by the lower rate of catalase synthesis in protein deficient animals.

In collaboration with Dr. Z. Hruban of the University of Chicago several characteristic hepatomas of different growth rates and catalase content were examined by electron microscopy and the enzyme contents of the tumors were measured. Microbodies were positively identified in the HC (high catalase), LC (low catalase), Reuber H-35 and Morris 5123 hepatomas but were completely absent from fast-growing Novikoff and Morris 3683 hepatomas. Examination of the data indicated that only hepatomas with a relatively high catalase and uricase activity contain microbodies and the morphology of the microbodies were quite characteristic. For example, in the Reuber H-35 hepatoma, the inclusions were large and contained large crystalloids whereas the 5123 hepatoma had small microbodies and the matrix was electron dense. The LC and HC hepatoma-microbodies were intermediate in size but were distinguishable on the basis of the small crystalloids which they contained. Good correlation has been found between the activity of uricase and the presence of microbody crystalloids with the highly characteristic crystalloid structure being shown by electron microscopy to be identical with the structure of purified uricase crystals.

The use of catalase as a genetic marker in C3H mice has been described previously. Recent surprising finding of a high catalase level in one of the C57BL substrains has prompted a thorough study by Dr. M. Reichcigl, Jr. Dr. W. E. Heston and Dr. H. A. Hoffman (Laboratory

of Biology, NCI) of the liver and kidney catalase activity of various C57 strains and substrains and of strain C58 all of which were originally derived from the Lathrop stock. No variation was found with respect to kidney catalase activity, however, the variation in liver catalase activity in certain substrains has suggested that a mutation resulting in a low liver catalase activity must have been present in the original Lathrop stock and that later reverse mutation to high level had occurred in two of the substrains. After a thorough genetic analysis reported elsewhere, the difference in liver catalase activity was shown to be due to a single gene difference, surprisingly with the low catalase level of activity dominant over the high. This gene had no apparent effect on the level of kidney catalase activity.

LABORATORY OF BIOLOGY

During the year the Laboratory of Biology has maintained its usual high productivity in basic areas of research as they relate to cancer. A total of 30 original papers have been published by the various members of the staff. Areas of immunology, biochemical genetics, and cell physiology and nutrition have received special emphasis but some of the work has also been in areas of virology, endocrinology, mammalian genetics, biochemistry, and tissue culture instrumentation and methodology.

The year has been marked by changes in personnel and program emphasis. Some of the older staff members are dropping out and younger members with new interests are being added. Dr. Charles Nicoll has joined the staff to strengthen the area of endocrinology. Dr. Daryl Granner who is also interested in endocrine problems and Dr. George Lyon who is in the area of biochemistry have been added to the Laboratory as Research Associates. Dr. Melvin Reuber, a pathologist, is being transferred into the Laboratory to strengthen the study of liver carcinogenesis—particularly of the spontaneous hepatomas of the mouse. Dr. Eva Leckband has joined Dr. Law's staff as an NIH fellow in his work on the role of the thymus in immunologic response. Dr. Ettore Appella, a protein chemist, is expected to join Dr. Potter on September 1, in their very ener-

getic program on the differentiation of myeloma immunoglobulins. We hope to bring in Dr. William Hall, an electron microscopist, on September 1 to fill this critical need of the Laboratory. Dr. Ram Parshad, a cytogeneticist, is joining the Tissue Culture Section in October as an International Fellow. In addition, three new Research Associates are joining the staff on July 1. While this spirit of youth taxes the budget it certainly envigorates the research program.

One of the greatest honors for the Laboratory this year was for Dr. Law to have been chosen to give the Clowes Memorial Lecture, thus recognizing him as one of the most outstanding investigators in cancer research.

The following summary includes studies chosen to illustrate the program of the Laboratory.

Immunology

Dr. Law has made a number of new observations in his study of the role of the thymus in immunogenic response. After neonatal thymectomy NIH (Swiss-Webster) mice do not show signs of the "wasting" syndrome found in other strains. A survey for a number of contaminating viruses showed no differences between NIH strain and the other strains that do "waste". However, in his study of the effect of neonatal thymectomy of germ free mice, Dr. McIntire showed the necessity of infection for the wasting syndrome.

Law has shown that the humoral-thymic factor for immune response is not species-specific because grafts of Osborne-Mendel rat thymic tissue can restore the immune response in neonatally thymectomized (BL \times C3H) F₁ mice.

The thymus is thus linked to oncogenesis. Law has shown that neonatal thymectomy strikingly increases the sensitivity of C57BL and C3H/Lw mice to the oncogenic potential of several strains of polyoma virus. Resistance can be restored with thymic grafts and inoculation of spleen and lymph node cells but not of spleen cells from thymectomized mice. The existence of an immunologic mechanism of the homograft type responsible for the resistance to polyoma virus oncogenesis is indicated by the work of Law and Ting. However, there was

no evidence of such resistance to the mammary tumor virus or to methylcholanthrene carcinogenesis for thymectomized C3H mice had fewer mammary tumors than controls and were not more susceptible to the induction of fibrosarcomas with MCA. The effect of thymectomy on oncogenesis by other viruses and in other species is being investigated.

Dr. Ting has demonstrated maternal transmission of antibody against polyoma virus. The milk was the route of transmission. He has also demonstrated transplantation resistance to Moloney lymphoma induced by two other murine leukemia viruses, PLLV and L-180 isolated in his laboratory. His results indicate that lymphoma induced by these three strains of virus share a common antigen.

Miss Uphoff has continued her work on the immune response, using radiation to study the host-versus-graft reaction and the graft-versus-graft reaction. She has also demonstrated genetic factors that influence irradiation protection by bone marrow.

Virology

Sanford has been trying to induce the malignant change in cells in vitro with Polyoma virus but whereas the virus infected cells appeared to transform earlier, the control cells also transformed. However, in the virus infected cells the transformation occurred long after the virus infection. Cell morphologic changes occurred but could not be correlated with anything except cells that were treated with high multiplicities of virus showed a delayed morphologic response. She also tested 5 strains of mouse and hamster cells for their capacity to develop polyoma-induced transplantation antigens in vitro. Such antigens were induced but their formation appeared to be a separate, independent phenomenon from the neoplastic change. Dalton has observed C particles in several of Sanford's cell clones in vitro but they differed from mouse leukemia viruses and produced no lesions when assayed in newborn mice of several strains. When the clones were adopted to the chemically defined media the C particles disappeared.

Dr. Ting has demonstrated the presence of viral genes in virus-free polyoma tumor cells.

Polyoma tumor 4198 originally induced in C3H with polyoma virus but now with no demonstrable virus was superinfected with SE 3049 virus. SE 3049 virus that had been grown on 4198 could then protect C3H mice against 4198 transplants although SE 3049 grown on normal mouse embryo cells gave only weak protection.

Dr. Law is continuing his study of congenital transmission of a leukemogenic virus, MLV. Three strains, C3Hf/Gs, C3Hf/Lw, and RFM have now been maintained through 15 generations as high leukemic strains owing to the transfer of the MLV through the mother's milk. Occasionally however, sublines will revert to low-leukemic lines because of failure of the MLV to be transmitted. There is no evidence of contact infectivity of MLV.

Dr. Andervont has continued his studies of the mammary tumor virus. In strain RIII he has noted that its effectiveness was decreased in certain females and did not regain its activity in many subsequent generations of descendants. When C3H mammary tumor virus is put in RIII mice it can also lose its effectiveness in certain females. When the RIII mammary tumor virus is introduced into C3H it does not increase in potency.

Dr. Barrett, in collaboration with Dr. Deringer, has been studying the latency of the mammary tumor virus to see if it represents a period of maturation of the virus-host relation or is primarily a maturation of the tissue susceptibility. He observed that the length of the latency period was the same whether the virus was injected into newborn test females, those one month old, or those 10 months old which indicates that latency represents a maturation of host-virus relationship.

Biochemistry

The discovery of feedback control over the uridine kinase reaction by Dr. Anderson and collaborators constitutes the first demonstration of such control over a salvage pathway for pyrimidine ribonucleotide biosynthesis. The control is very effectively exerted by the end-products CTP and UTP, and the mechanism of the inhibition has proved to be exceedingly interesting. Feedback control of the kinase has

been found in a variety of cell types. However, in microorganisms that utilize uracil, a similar kind of feedback control is exerted, instead, on UMP pyrophosphorylase. The pyrophosphorylase is probably the major salvage pathway of biosynthesis in these cells, whereas the kinase pathway predominates in other organisms, including the mammalian cell systems. Studies (mostly by Dr. Lyon, a research associate here since July 1964) are also being made on uridine phosphorylase, using preparations from both microorganisms and mammalian tissues; the studies are aimed at defining whether this enzyme serves an anabolic or catabolic role in metabolism and whether this role determines the properties of the enzyme, including its substrate specificity and response to control mechanisms. In connection with our studies on the sites of action of antimetabolites, careful and extensive work has been done (by Mr. Ciardi) on the identification of various purine and pyrimidine metabolites. These have included pioneer use of the new and versatile method of thin-layer chromatography.

Biochemical Genetics

Dr. Potter who recently has been collaborating with Dr. Appella has continued his very active study of myeloma immunoglobulins in BALB/c mice with plasma cell tumors. The transplantable plasma cell tumor is like a clone of cells in respect to protein secretion and BALB/c mice with these tumors have large quantities of specific myeloma immunoglobulins in their serum and urine. This is the best means of separating in relatively pure form specific immunoglobulins from the heterogeneous family of immunoglobulin molecules that exist in the normal individual. The light and heavy polypeptide chain subunits isolated from a series of myeloma proteins were classified by antigenic structure, tryptic peptide maps, and amino acid composition. It was found that there were two types of light chains, kappa and lambda, and five types of heavy chains, alpha, mu, gamma F, gamma GBe-1, and gamma GBe-2. Thus seven structural genes which participate in immunoglobulin formation in the inbred BALB/c mouse have been identified. When the heavy chains from an individual

class for example gamma F were compared it was found that there were differences in the amino acid and tryptic peptide map composition. Similar differences were found among gammaGBe-1, gammaGBe-2, and gamma A heavy chains. These findings have implications in the mechanism of the chemical basis of antibody variation.

Potter has studied the genetic control of heavy chain polypeptide synthesis in collaboration with R. Lieberman and S. Dray of NIAID. Utilizing myeloma proteins and allotypic precipitating antibodies prepared by immunizing mice of one strain with immunoglobulins of another it has been shown that mice of the a4 allotype group differ from the a1 BALB/c in the gammaGBe-1 heavy chain. Genetic linkage of the gammaGBe-1 and gammaGBe-2 heavy chain specificities has been demonstrated.

Dr. McIntire in studying the Bence Jones proteins has demonstrated that a group of them not previously known to function as a light chain are definitely found as the light chain of a myeloma macroglobulin and are also found by antigenic testing in the macroglobulin of normal mice. This chain now designated as the kappa chain is found in immunoglobulins other than macroglobulin in strains of mice other than BALB/c. This means there are at least two antigenic types of light chains in mouse immunoglobulins, the other being lambda or the L chain, which corresponds to the number of types for human immunoglobulins. Special study with Kuff of a macroglobulin produced by one of his plasma cell tumors indicated that the macroglobulin, like other myeloma globulins of mice, is composed of 2 different size polypeptide chains (heavy and light) linked by disulfide bond.

In studies of starch gel electrophoresis patterns of urine of male and female mice of 18 inbred strains, Dr. Hoffman has found 6 different electrophoretic patterns, two different 3-band patterns, and four different 4-band patterns. From genetic analyses the 3- or 4-band electrophoretic mouse urinary protein types appear to be under the control of a pair of co-dominant alleles. However, the locus for the MUP types appears to be complex with at least 6 alleles represented in the 18 inbred strains.

Hoffman has also been studying serum protein variants or serotypes. A serum protein has been discovered in BALB/c that is antigenic to the NBL strain. From immunochemical studies it appears that the protein is probably a beta-globulin with a high molecular weight. Genetic linkage studies of this protein with the immunoglobulin Ig-1 trait, the low liver catalase Ce trait, and a variant of caracal coat gene Ca are in progress.

Mammalian Genetics

In their studies of effects of specific genes on occurrence of tumors, Heston and Vlahakis have demonstrated that the newly discovered mutation viable yellow A^v greatly increases susceptibility to mammary tumors. Like its allele, lethal yellow, it also increases occurrence of pulmonary tumors and hepatomas. A^v arose in strain C3H and the sublime with this gene, C3H-A^v, is now the most susceptible strain we have to mammary tumors and hepatomas. In contrast, the dwarf gene that stops growth at 7 to 10 grams completely inhibits mammary tumors, hepatomas, and urethan induced lung tumors except for a few very small lung tumors that do not grow.

The very high incidence of hepatomas in strain C3H, especially C3H-A^v and their hybrids, is of special significance. In C3H-A^v males hepatomas occur in 100 percent of the animals and each animal has many hepatomas. This now provides excellent material for further study of factors in the etiology of this neoplasm.

Dr. Deringer has reported an incidence of 22 percent of mammary tumors in her BALB/c females in contrast to the extremely low incidence reported by others. This is probably owing to her maintaining them in a healthy condition to a much greater age and observing them more closely. She also noted 10 percent with reticular neoplasms. This is of significance since BALB/c is used so extensively as a test strain. She has also recorded the occurrence of amyloid in organs of strain BL/LyDe mice. This would be an excellent strain for studying amyloidosis. Deringer has reported an increase in reticular neoplasms in her DBA/2eB mice

with urethan. She had also reported an increase in hemangioendotheliomas and has now shown that these are transplantable.

Endocrinology

Dr. Nicoll joined the laboratory September 1, 1964 and has now gotten underway a rather extensive endocrinology program including growth of the mammary gland and the role of the stroma which he has demonstrated to have an important regulatory action; in collaboration with Hoffman comparison of secretory action of normal and neoplastic mouse pituitary gland using organ culture and subjecting the medium to starch-gel electrophoresis; an evolutionary study of prolactin; and the development and modification of the pigeon crop-sac assay of prolactin. During the year he has also published several papers on work done previously.

Dr. Granner, who joined the Laboratory in July 1964, is making a descriptive study of the premalignant mammary plaques in strain DD mice. He is also studying the effect of progesterone as well as estrogen in induction of mammary tumors in strain A mice.

Cell Physiology and Nutrition

Dr. Evans and co-workers have adapted a number of additional cell lines including C3H mouse kidney, C3H mouse embryo, and green monkey kidney cells to chemically defined media and this media supplemented with horse serum and with calf serum. On the basis of growth of intraocular transplants the time of the malignant transformation is fixed with remarkable biological reproducibility. One of the most outstanding findings is that cells in the media with calf serum transform much later (if at all) than those in the media with horse serum. Studies are underway to try to determine what this difference between horse serum and calf serum in producing the malignant transformation is due to. The foetal calf serum delays but does not arrest alterations in chromosomes.

For the first time non-malignant freshly explanted mouse cells have been grown in chemically defined media in large enough quantities and good enough quality to be used in nutri-

tional and transformation studies. Dr. Evans has now shown that C3H mouse embryo cells explanted directly into the chemically defined media with absolutely no added serum or any supplement will undergo the malignant transformation in approximately 130 days. A considerable list of biochemical and nutritional differences in cells *in vitro* have now been recorded by the tissue culture group.

Evans has shown that HeLa, H.Ep.2, a hamster cell strain, and one mouse embryo cell strain all require both vitamin B₁₂ and i-inositol. The liver I strain may require both vitamins A and D. The green monkey kidney and liver II strains appear to be less specific than the other strains in requirements.

Sanford had noted consistently higher uptake of glucose and production of lactate in a high-tumor producing clone of cells than in a low-tumor producing clone. However, inoculum size was variable and when its effect was tested it was found that higher uptake of glucose and production of lactate occurred in clones grown from low inoculum size than from high.

Dr. Westfall has made a number of observations of five enzymes in cells on chemical defined media. For example, cells of two independent adaptations of a clone of mouse liver cells to chemically defined media showed different arginase activities. One continued to show high arginase of the progenitor line while that in the other dropped. In another study he noted that cells grown in media supplemented with calf serum had twice to 3 times the lactate dehydrogenase as those in media supplemented with horse serum. These biochemical markers are useful in characterizing differences cells undergo in long-term cultivation *in vitro*.

Sanford had made the interesting observation that when she clones cells of Swarm's teratoma they lose their ability to differentiate. This was noted in 7 clones, whereas parental lines not cloned maintained this ability. This seems to answer a question that has been asked of teratoma cells for many years.

In cooperation with Tjio and Wang, Sanford has been trying to hybridize cells in culture. Using chromosomal markers they as yet have no evidence of hybridization and thus are not able to confirm the work of Ephrussi and others.

Tissue Culture Methodology

Dr. Bryant has made further studies on the shaker flasks for cultures. He now has H.Ep.2 and mast cell 815 in chemically defined media in the shaker flasks. The larger the flask the lower the concentration of cells and the stress on the cells in the medium sized flask is not enough more than the smaller flask to prevent their use. Therefore the medium flask is the choice for building up high cell populations suitable for the continuous flow culture. This has been done with line 2071 cells.

Mr. Andresen is being successful with the mass stirrer flask. This permits continuous nutrient fluid renewal and continuous withdrawal of cells. This is the only place this can be done. Commercial organizations still have to use the batch system.

Both Evans and Sanford have been testing several commercial media qualitatively and quantitatively but none compares favorably with their own media.

The motion picture camera is being used successfully to determine growth and mitotic rates in cells in the chemically defined media.

PATHOLOGIC ANATOMY BRANCH

In addition to the diagnostic pathology services performed in the Department of Pathologic Anatomy, the permanent staff and residents study and make reports on human case material and perform a variety of types of research. Most of the specific animal and laboratory research projects of the staff have been mentioned earlier in this summary, so the principal items listed here will be those performed by the resident staff. Many of the studies of human material are in collaboration with clinical staff or with staff of the pathology department.

Dr. Berard published a number of articles this year on experimental work he performed at Walter Reed Army Institute of Research. These included studies on the effect of ascorbic acid and deuterium oxide on wound healing. In addition he has studied a number of diseases in man including: clostridial septicemia in patients with cancer, disseminated histiocytosis, the central nervous system in patients with congenital heart disease, the hypothalamus in a

patient with acute intermittent porphyria, a defect of antidiuretic hormone activity, ocular pathology in patients with leukemia, coarctation of the aorta with ventricular septal defect. In addition he is collaborating with Dr. Gittes on the effects of pineal extract on the testis of rats and with Dr. Malmgren on immunologic responses in tumor-bearing mice.

Dr. Chambers has studied patients with Sjogren's disease, alopecia and a malignant lymphoma and has been attempting to develop a line of African lymphoma cells adapted to Spinner culture. In addition he is collaborating with Dr. Gelderman in studying the effect of EDTA and mitomycin on experimental melanoma in tissue culture.

Dr. Gelderman has published several articles on LDH isozymes this year. One of these is as a chapter in a book by Sunderman and Sunderman, *The Serum Proteins and the Dysproteinemias*. In addition he is doing several experiments with EDTA and mitomycin designed to elucidate the molecular basis for cancer therapy. He is studying cases of fibrocystic disease of the pancreas and of the cerebellar changes in patients with leukemia.

Dr. Grimley has continued developing techniques for using light microscopy and electron microscopy on the same tissue. He is also studying human cases of cymomegalic inclusion disease, carotid body tumor, and the association of glioblastoma with other tumors.

Dr. MacLowry has been studying the juxtaglomerular apparatus in experimental hypertension and spleno-renal shunt in dogs. In addition he is studying patients with spermatogenic arrest following chemotherapy and is helping establish a data retrieval program for the Surgery Branch. He also has reviewed the lungs of mice inoculated with T241 sarcoma and with ethiodol and poppy seed oil.

Dr. Wright with Dr. Grimley published a paper on *Torulopsis glabrata* infection in man. He is currently working on a number of human diseases, including congenital anomalies of the entire autopsy population, cerebellar changes in patients with leukemia, conduction defects in patients with calcific aortic stenosis and mycosis fungoides. He also is working with Dr. Thomas on a study of immunity in mice inoculated with L1210 leukemia.

LABORATORY OF PATHOLOGY

Introduction

The work in pathology falls into two general categories: the work in the Laboratory of Pathology and the work in the Department of Pathologic Anatomy. The staff of Pathologic Anatomy performs the autopsies, examines the biopsies and surgical pathology specimens and does exfoliative cytology for patients in the Clinical Center.

The primary aim of those in the Laboratory of Pathology is to carry out experimental cancer research and for this work they use a variety of techniques and experimental animals. Each member carries out his own experiments, two or more members collaborate with each other and individual members collaborate with others in the National Cancer Institute.

There is free exchange of information and help between and among all the pathologists. While the Annual Report reflects in general the work of the two groups in pathology, it also shows that there is considerable interchange, and interchange is constantly in progress. Most of the members of the Department of Pathologic Anatomy have under study one or more problems involving cancer in man or some disease in man which they carry out either independently or in collaboration with the clinicians. The autopsies and the surgical pathology specimens and biopsies are examined with the greatest of care. They are worked up by residents and their diagnoses are all reviewed by senior staff members.

A notable accomplishment contributed by the head of the Department of Pathologic Anatomy, Dr. Louis B. Thomas, is his work on a committee of the College of American Pathologists which has devised a nomenclature and code for pathological lesions. By the use of this the pathological material from the human cases have been arranged for data processing, and it is now in operation.

Most of the members of the Department of Pathologic Anatomy also carry out experimental research using animals, tissue culture and other special techniques. The residents in Pathologic Anatomy are particularly ambitious in carrying out research programs.

Experimental

The experimental work in the Laboratory of Pathology is not restricted to a single project, or to a group of closely related projects, but each pathologist follows his particular line of interest and training. Because of this diversity the projects in the laboratory have been separated into 5 rather loose categories for convenience in preparing this summary. These will be:

Cancer in Man, and Related Animal Studies

- A. Geographic Pathology of Cancer
- B. Exfoliative Cytology
- C. Possible Etiologic Factors in Human Environment

Cancer in Animals

- A. Pathogenesis
- B. Modifications in Carcinogenesis
- C. Biologic Factors in Neoplasia
- D. Development of Spontaneous Tumors

Viral Carcinogenesis. Problems of immunology and resistance.

- A. Polyoma Virus
- B. SV40 and Adenovirus
- C. Leukemogenic Viruses
- D. Immunology and Resistance

Accumulation of Data. Laboratory animals and other species.

- A. *Information on Laboratory Animals*
- B. *Phylogenetic Aspects of Neoplasia*

Collaborative Research

- A. Methods
- B. Examples

CANCER IN MAN AND RELATED ANIMAL STUDIES

Since all but one of the members of the Professional Staff of the Laboratory of Pathology and Department of Pathologic Anatomy are medically trained, many research activities are correlated with the problem of human cancer. Several projects are directly utilizing human material, and others are testing in animals substances in the human environment that may be carcinogenic.

A. *Geographic Pathology of Cancer.*—Information is accumulating that certain forms of cancer in human population groups occur in a

higher incidence than would be expected. Intensive study of such groups, and the recognition of possible etiologic agents which can be tested on experimental animals may identify some environmental factors which could be eliminated or controlled and thereby prevent many cases of human cancer. The following studies have this aim:

1. Maps of different geographic areas are in preparation to show the relative frequency of different types of cancer. When it is recognized that some type is especially common, a more intensive study of the population group is indicated. (Dunham and Bailar)

2. The following types of cancer are being investigated in particular geographic areas:

a. Bladder cancer in New Orleans. White males over 60 in this city are frequently affected. All histologic sections have been reviewed by the same pathologists (Stewart and Rabson) to establish uniformity in diagnosis. Questionnaires on past history and possible exposure to carcinogens are being analyzed. (Dunham)

b. Uterine cancer in New York City, Israel and Washington, D.C. This type of cancer is notably frequent in Negro women and infrequent in Jewish women. Histologic sections have been examined from patients with cancer, and over 3500 women interviewed. The data will be coded in an effort to detect an environmental factor that may account for the racial difference. (Stewart, Dunham, Thomas, Edgcomb). A survey of uterine cervical cancer in Colombia, S.A. where the incidence appears to be high, is in progress. (Malmgren) (See next page).

c. Lung cancer in Veterans of World War I. The medical history, and biopsy and autopsy material from this group is generally accessible. Histological material has all been reviewed by the same pathologist, and a uniform classification of histologic types has been made. This survey indicated the unreliability of random histologic diagnoses which have often been accepted uncritically by statisticians or epidemiologists. All available cases have now been reviewed, and the results are being coded. This study will supply important information on the influence of smoking on the incidence of lung cancer within this group. (Herrold)

d. Gastric Cancer in Japan. Cancer of the stomach is remarkably frequent in several unrelated human populations, and in Japan, this type of cancer is especially common. Migrant Japanese in Hawaii offer a good group of genetically similar people in a different geographic area for comparison. The histologic sections are being reviewed by the same pathologist (Herrold), and any association with gastric ulcer, polyps or intestinal metaplasia is noted. A correlation will be made with data collected by a team of epidemiologists. An investigation is also made for viruses (Bryan); and electron microscopy and histochemical information is obtained when possible. This is a long term study that will require many years, but a preliminary report will be given at the International Congress in Japan in 1966. (Herrold, Stewart, pathologists from Japan)

e. Malignant lymphoma in the U.S. states and Africa. The frequency of this form of cancer in children in Africa has aroused much speculation as to possible etiologic factors. It became important to determine whether this malignant lymphoma of unusual histologic type and anatomical distribution was restricted to Africa or whether a similar neoplasm occurred in other geographic areas. Histologic study of cases in the U.S. revealed that $\frac{1}{3}$ of the U.S. cases were of similar histologic type, but the disease is rare in comparison with Africa and manifestation in the jaw is infrequent. In the U.S. the relative numbers of the specific histologic types of malignant lymphomas were found to differ in children and in young adults. The conclusion was reached that the tumor in Africa is an "incidence" phenomenon, and not a unique type of neoplasm. (O'Conor, NCI, Smith, AFIP, and Rappaport from University of Chicago)

Additional studies were made on a case brought from Africa to the Clinical Center. No particles were revealed on E.M. study of the original tumor, but particles were found after several transfers in tissue culture. This confirmed other work performed with tissue from this case at the National Cancer Institute. (O'Conor, Rabson)

B. *Exfoliative Cytology*.—A diagnostic service in exfoliative cytology is supplied to the Clinical Center, but in addition to this, a

number of research studies are in progress in collaboration with various clinical services. These relate to the presence of neoplastic or cancer patients is not closely correlated with other cells on body surfaces or in fluids, and sex chromatin in exfoliated cells. It has been determined:

1. Number of neoplastic cells in the blood of metastasis, but a possible increase of megakaryocytes in blood in patients with cancer is found.

2. Cancer cells are often recovered from wound washings after cancer surgery, but time will be required to determine whether the frequency is correlated with recurrence in the wound site.

3. Leukemic cells in the spinal fluid provide a reliable indication of central nervous system involvement and assessment of therapy.

A comparison of cervical cancer in Connecticut and Southwest Region of England has been reported. (Thomas and Bailar) Additional reports from this survey will include a correlation of 5-year survival rates with the histological features of the cancer found in the two areas and a detailed description of the cancers found among women in the two areas.

4. An improved technique for identification of sex-chromatin in cells from the buccal mucosa has been developed, and it appears that there is a correlation between the hormonal status in the female and the sex chromatin count, since a decrease in sex chromatin was found at the time of ovulation.

5. The reported presence of a "malignant associated change" in cells of the buccal mucosa was not confirmed.

6. A technique for identifying cells from mesothelial surfaces by E.M. study is being prepared.

7. A method for coding findings in exfoliative cytology is being prepared. (Malmgren)

C. *Possible Etiologic Factors in Human Environment.*—1. Adsorbates from drinking water from New Orleans, where bladder cancer is high, and Birmingham, where it is low, are concentrated and given to mice. Betel quid ingredients and tobacco snuff are being tested in the cheek pouch of hamsters. DMSO (dimethyl sulfoxide), a chemical long used as a commercial solvent and lately proposed as a

therapeutic agent because it readily permeates the skin, is being investigated as a possible carcinogen in mice. No results can yet be reported from these experiments because the effect of weak carcinogens such as some of these may be difficult to assess, and requires a long period. (Dunham)

2. A study on the effect of two strains of *Schistosoma haematobium*, Gold Coast and Egyptian, on the hamster are continuing. This organism is associated with bladder cancer in Egypt but not in Africa. Histologic study is not yet completed. (Thomas, Berry).

3. Thorium dioxide, once used as an x-ray contrast medium, has produced vascular neoplasms of the spleen in guinea pigs two years after administration. It is hoped that a dose effect demonstrated may be studied in greater detail in guinea pigs. (Swarm)

4. Numerous other substances to which human beings are exposed have been tested, or are now being tested but the results so far are negative or incomplete. Material from an ochre barrel of African natives with a high incidence of carcinoma of the esophagus produced no cancer, and dihydrosaffarol (DHSF) a food coloring is negative so far. (O'Gara). Experiments with metals such as zinc, copper, iron, and heated fats given to rats are incomplete. (Hueper, O'Gara). Possible carcinogenicity of Cycad meal given to mice is incomplete but degenerative changes have been found in the liver. (O'Gara). Although a procedure effective in demonstrating pulmonary carcinogenesis from known chemical carcinogens was used, beryllium oxide, chromium oxide, asbestos, air pollutants and tobacco tar, trapped in infarcted areas of the lung failed to produce neoplasms in rats. (Stanton). A number of substances mostly hormones were injected into newborn mice, but so far only estrogen proved to be carcinogenic, and produced cervical cancer after two years. Progesterone produced no alteration but enovid given to newborns has caused lesions in the uterine cervix 15 months later that may be preneoplastic. (Dunn)

5. Possible relationships of cancer in man with acanthosis nigricans. A tissue culture line has been established from a uterine cancer in a human being who also had acanthosis nigri-

cans. Cells from the culture were transplanted to the hamster cheek pouch. No melanocyte stimulating hormone was found. PPLO found earlier in the cell cultures was apparently a contamination of the test bacteriological cultures. The line is being continued, but no further work is now contemplated. (Dawe) (See #6)

6. Pulmonary changes in patients with cancer. Dr. Powell has developed a machine to inflate human lungs under conditions of constant pressure and flow. He is using this to make a detailed study of pulmonary changes in patients with leukemia and of patients with emphysema.

CANCER IN ANIMALS

Many of the studies of cancer in animals are concerned with: A) Pathogenesis, or the steps by which cancer develops after exposure to a carcinogen. B) Modifications in the activity of carcinogens produced by altered conditions or substances. C) Biologic factors of neoplasia such as the behavior of tumors during transplantation, metastasis, and effects of irradiation, and D) Spontaneous, transplanted and unusual tumors.

A. *Pathogenesis*.—1. Development of tumors produced by 2-7-FAA when given to pregnant, and lactating mothers, to newborns, and to adult rats is being studied. The distribution of skin appendage tumors produced by this chemical is being plotted on maps of the skin surface and a paper on histogenesis is being prepared. The effect on young rats requires further time to determine. (Stewart and Snell)

2. An intensive study is in progress on the histogenesis of liver cancer produced by N-2-fluorenyldiacetamide. The carcinogen is given at various dose levels. Early hyperplastic lesions failed to grow when transplanted to the liver, but later ones grew and retained their histologic appearance. A histologic study is also being made of hepatomas in hybrid and inbred mice with the yellow gene. This detailed study of a single form of cancer may identify more precisely the point at which the neoplastic alteration occurs. (Reuber)

3. Nitrosamines were found to be potent carcinogens, and were reported to produce brain tumors in rats. Nitrosamine compounds given

by a variety of routes in hamsters produced tumors at similar sites, indicating that the distribution, metabolic pathways, and excretion of the carcinogen were the critical factors. Tumors of the olfactory neuroepithelium in Syrian hamsters were of especial interest, because in previous reports these may have been mistaken for brain tumors by others. These studies emphasize that carcinogens may reach remote sites, and that when the site of tumor formation is in the lung, the carcinogen did not necessarily reach the lung by inhalation. (Herrold)

4. Syrian hamsters were given benzopyrene by intratracheal instillation and early changes in the bronchial epithelium were described. No tumors resulted from atmospheric pollutants or tobacco tar. An unanticipated finding from this experiment was the development of cryptococcus neoformans meningitis in one hamster. This disease occurs in man, where it has been presumed that the organism is blood-borne from a focus in the lung. Observations with the hamster suggest that the primary focus may be the nasal cavity and sinuses, from which sites the infection extends to the meninges. (Herrold)

B. *Modifications in Carcinogenesis*.—1. The effect of a carcinogen may be altered by the age of the host when it is given. Newborns are especially susceptible and some carcinogens pass the placental barrier. Nitrosamines given to pregnant rats caused tumors in the offspring. The monkey is not inherently refractory to carcinogens, since hepatomas have been produced after nitrosamine. This is the first that hepatomas have been induced in monkeys and the first that a procedure has consistently produced tumors in monkeys. (O'Gara, Kelly)

2. The oncogenic response to carcinogens is increased in the lung if the pulmonary artery is ligated and an infarct produced in which the carcinogen appears to be trapped. (Stanton)

3. Oncogenesis of the uterine cervix in hamsters produced by DMBA is inhibited if Vitamin A is given. (Chu)

4. Special diets have a protective effect against carcinogens, but this can be overcome by larger doses of carcinogen. (Mulay)

5. A strain of rats which carries a gene for congenital hyperbilirubinemia with jaundice is

under study. A decrease in glucuronyl transferase is found, and a similar condition is found in some human beings. An effect to inbreed these rats is making progress. A study on the effect of a chemical carcinogen, N-2-fluorylacetamide, is incomplete. Transplantation of a hepatoma shows variation in the number of lung metastasis; none appear in the non-jaundiced rats, but they are numerous in the jaundiced. (Swarm)

C. *Biologic Factors of Neoplasia.*—1. Transplantation of a murine chondrosarcoma outside of the inbred strain of origin was successful in a well-differentiated tumor, and this may be due to the protective effect of the cartilage matrix. Cartilage survived after transplantation of a teratoid testicular tumor when other tissues were destroyed by the host, another indication that the matrix protects from defense reactions of the host against foreign cells (Swarm) Clones of cells from the teratoid tumor obtained by tissue culture did not retain totipotency. (Sanford, Swarm)

2. Metastasis of a mammary tumor was increased in C3H mice when orotic acid was given. Thus a fundamental biologic factor in a neoplasm can be altered by a substance given to the host. (Chu)

3. Irradiation of a chondrosarcoma produced the same effect if it was administered 24 hours before transplant, as when performed 24 hours after transfer. This offers a means for studying the primary effect of irradiation on a tumor and avoids the effect of irradiation on the host. (Swarm)

D. *Development of Spontaneous Tumors.*—Studies on these tumors are important to determine the natural incidence and pathogenesis.

1. Spontaneous tumors are studied in various inbred strains of rats and in mastomys. The incidence of gastric cancer in mastomys appears to be genetic, and differs in 2 strains under study. (Snell)

2. A number of transplanted tumors are maintained and are available to others. (Snell, Dunn, Swarm)

3. An unusual contagious tumor in hamsters has been studied intensively in the past few years. This tumor is a reticulum cell sarcoma with leukemic manifestations, and can be transferred to other hamsters by feeding tu-

mor tissue or cage contact. Recently the tumor was transferred by the bite of mosquitoes. It was proved that the mosquito transferred living cells and not a virus. This opens a new possibility of cell transfer by insects, rather a viral transfer. (Banfield)

VIRAL CARCINOGENESIS

Problems of immunology and resistance. The same interest in pathogenesis and the mechanisms of carcinogenesis will be found in the experiments carried out by pathologists with viruses as was noted in experiments with chemical carcinogens. Experiments now in progress are concerned with: A) Polyoma virus, B) SV40 and adenovirus 12, and C) Leukemogenic viruses. Other studies are also in progress on D) Immunology of virus tumors and E) Interferon.

A. *Polyoma Virus.*—1. Studies on the interaction of mesenchymal and epithelial elements in organ culture, and the effect of the polyoma virus have continued. Major findings are that the submandibular gland whether rudimentary or adult will develop neoplasms if polyoma is added to the culture, but salivary gland mesenchyme is necessary for the neoplastic change in the epithelial cells. Although hair-follicle tumors are found in mice with polyoma, hair-follicles to new syngeneic hosts remained in the growth phase of the cycle and did not develop tumors. (Dawe) Tooth buds infected by polyoma and transferred to subcutaneous sites developed ameloblastomas. (Main, Dawe)

2. After polyoma infection tumors found in the nasal fossa were proved to be from the neuroepithelium. These tumors were similar to, but less invasive than the neuroepitheliomas induced in hamsters and rats by diethylnitrosamine (Herrold) and by 2,7-FAA (Snell) respectively. Since nasopharyngeal tumors are common in parts of Indonesia, information on pathogenesis at this site is of interest. (Dawe)

3. Polyoma virus has produced tumors in the brain of newborn hamsters. Relation between growth of virus and tumor induction has been determined. (Establé, Rabson)

4. Hemangiomas induced in hamsters by polyoma are transplanted with difficulty in the first generation, but success increases in later

generations, and one line metastasized consistently. In early generations, the takes were less successful in polyoma infected hosts, but this resistance was overcome in later generations. Fish have been exposed to polyoma but no results can be reported as yet. (Stanton)

5. The behavior of a polyoma induced tumor in the hamster was compared with a spontaneous and a carcinogen induced tumor after intravenous injection of dissociated cells. The resulting nodules in the lung after injection of the polyoma tumor were interpreted as inflammatory reactions rather than true neoplasms. (Herrold)

6. Thymectomy was shown to increase the oncogenic effect of the polyoma virus. Of particular interest was the observation that strains of mice resistant to polyoma oncogenesis were made susceptible by thymectomy and that susceptible mice which do not respond to a strain of polyoma of low virulence did develop tumors when thymectomized.

B. *SV40 and adenovirus*.—1. Adenovirus growth in monkey kidney cells is enhanced by SV40. Other viruses tested failed to produce this enhancement. Adenovirus 12 induced tumors in C3H and BALB/c mice after thymectomy on day of birth. Cells from rhesus monkey were grown in tissue culture with Simian virus 40 and "transformation" noted, but cells failed to produce tumors when transferred back to the original donor. The possibility is suggested that the cells in culture had acquired an antigen from the virus, and that the monkey recognized this as foreign. These results indicate that infection with oncogenic viruses may be no risk in normal human beings, but a possible hazard exists in persons with reduced immunologic resistance as in thymectomized mice. (Rabson)

2. Adenovirus 12 in green monkey kidney produces a characteristic nuclear stippling cells which is associated with active viral replication only in the presence of SV40. Other virus combinations failed to produce this effect. (O'Conor) The growth of both viruses in the same nucleus has suggested that "hybridization" and genetic recombination may occur.

7C. *Leukemogenic Viruses*.—1. Moloney virus infection in rats has disclosed previously inapparent Bartonella and encephalitozoon or-

ganisms in the preleukemic state. (Stanton) Nucleic acids given preceding Moloney virus injection stimulated leukemogenesis. (Malmgren)

2. Rauscher virus has been given to mice infected with a malarial parasite that produces a severe anemia, and combined diseases will be studied. (Edcomb) Mice infected with the Rauscher virus survive longer than controls if given propylthiouracil. Thyroidectomy failed to produce this effect. (Dunn) A great prolongation of life occurred in mice given blood transfusions after Rauscher virus. (Malmgren)

D. *Immunology and Resistance*.—1. Virus induced antigens are found in transformed cells from tissue culture although two strains of polyoma with different oncogenic potential were similar in their ability to produce antigen, yet the more oncogenic strain which was also thymotropic when injected into newborn animals inhibited the animal's ability to reject heterologous tumor transplants. This could have been because of injury to the thymus. (Friedman)

2. A replicative form of an arborvirus has been found and characterized. It is now under study and should prove useful in determining the cellular location of RNA. (Friedman)

3. Mechanisms of interaction of interferon and possible role in carcinogenesis are under study. The action appears to involve active protein synthesis. Interferon appears to limit production of viral RNA. (Friedman)

ACCUMULATION OF DATA (LABORATORY ANIMALS AND OTHER SPECIES)

Because the training of pathologists is not restricted to one organ system or type of disease, they are often able to contribute to the general knowledge of the normal and pathologic anatomy of many different animal species. This contribution is often incidental to the main purpose of cancer research, yet it is indispensable to the intelligent use of biologic material. Unless the normal anatomy and spontaneous pathologic alterations are recognized, it is impossible to interpret the effects of experimental procedures.

A. *Information on Laboratory Animals*.—1. Information is continually being added on inbred strains of rats, and more recently on

mastomys. In this species the female has a well developed prostate gland, which will be described in a forthcoming publication. (Snell)

2. A guide to the normal and pathologic anatomy of the laboratory mouse is being prepared. (Dunn)

3. Complete autopsies are being performed on both control and experimental monkeys and much needed information is being gathered about these primates. A form of vascular disease resulting from subcutaneous injections of polycyclic hydrocarbons has been described. (O'Gara)

4. Collagenous connective tissue and elastin from the embryo to old age in man and laboratory animals is being studied. A new staining and extracting procedure for elastic fibers using KMnO₄ is being used in this study. (Banfield)

5. The maintenance of fish within the laboratory and use for experimental carcinogenesis has been accomplished. (Stanton)

6. The normal estrous cycle has been established by vaginal smears for Sprague-Dawley and Fischer rats, C3H mice, Swiss, and DBA mice, hamsters and guinea pigs, and the effect of constant light and darkness on the cycle in some of these animals has been determined. (Chu)

B. *Phylogenetic Aspects of Neoplasia.*—1. An important project has been started on phylogenetic aspects of neoplasia in cooperation with the Smithsonian Institute and Marine Biology. The collection and identification of neoplasms in invertebrates has been started, a literature survey now having over 300 entries will be continued and a registry of specimens will be made. Neoplasia in planaria and cockroaches is being investigated under laboratory conditions. It is already apparent that adequate criteria have not been used previously to determine neoplasia in lower forms. Tumors have been found in fish from a lake where no outboard motors were used, thus eliminating motor oil as the only carcinogen in fresh-water lakes. (Dawe)

COLLABORATIVE RESEARCH

It is recognized that many research projects at the National Cancer Institute require the col-

laboration of a pathologist, especially in the final evaluation of the effect of an experimental procedure on laboratory animals. The Laboratory of Pathology has always tried to make this assistance available.

A. *Methods.* 1. The pathologist may take an active part in planning an experiment and in following it through; he may take the responsibility for all autopsies and histologic diagnoses in an experiment; he may review only the histologic sections in a given experiment; or he may serve as a consultant to review selected material with no responsibility for the entire experiment or its publication. Finally, he may make use of material accumulated by other investigators for independent studies concerning pathologic alterations. It is emphasized that full collaboration of the pathologist at the time the experiment is planned is the most satisfactory arrangement for it insures the best and most economical selection of material for pathologic studies.

2. In addition to the use of the light microscope and standard autopsy procedure, individual members of the Laboratory of Pathology have become proficient in special techniques such as fluorescent antibody visualization, electron microscopy, tissue culture, autoradiography, exfoliative cytology, special cytology, and histochemistry. These special skills are often made available in collaborative studies.

B. *Examples.* It would be tedious to consider all the collaborative work now in progress in the Laboratory of Pathology, and this should be unnecessary since it is covered in reports from other laboratories. However the following are noteworthy:

1. Fluorescent antibody studies by Dr. Malmgren. He has studied a) plasma cell neoplasms and other conditions in man and animals in collaboration with Dr. Fahey; Dr. Solomon and Dr. Brecher; b) localization of antibodies in mycosis fungoides with Dr. Kenneth Blaylock; c) Antigens in human leukemic cells (with Dr. Mary Fink, Frank Rauscher, Henry Orr, and Myron Karon).

2. Dr. Dunn has continued collaborative studies with Dr. Andervont on mammary tumors; with Dr. Evans on the effect of transfer of tissue cultures to the anterior chamber of the eye;

with Dr. Law on thymectomized and leukemic mice; and has diagnosed sections for many other investigators both in and out of the NCI.

3. Dr. Banfield has used the electron microscope to assist Dr. Handler, Dr. Tani and Dr. Kuff with identification of cellular fractions; with instrument engineering to develop a tissue changer for E.M.; with Dr. McMaster to determine if antibodies against tobacco mosaic virus occur in man; with Dr. Steinberg and Challoner to correlate morphologic changes with the biochemical effects of a high fatty acid concentration and with Dr. Lester, Nadel and Reuber in E.M. investigation of normal and pathologic tissues. Findings are that macrophages persist in Whipple's disease; cytoplasmic crystals were found in presumed leukemic cell of guinea pig, but no virus; Fabry's disease was diagnosed by E.M., but the diagnosis was missed with light microscope.

4. Dr. Snell is frequently consulted regarding the normal and pathologic anatomy and neoplasms in laboratory rats.

5. Dr. Stewart frequently gives an opinion regarding lesions observed by other investigators at the NCI, and from outside.

6. Dr. O'Gara is becoming recognized as an authority on the pathologic anatomy of the monkey, and assists others in this field.

7. Dr. Reuber has collaborated with Dr. Harold Morris on studies of minimal deviation tumors.

LABORATORY OF PHYSIOLOGY

Office of the Chief

The mechanism of carcinogenesis by ultraviolet light is being pursued by Dr. Blum. He has been concerned with obtaining and analyzing data from experiments on epidermal hyperplasia of mouse skin induced by ultraviolet light. In counting numbers of epidermal cells and of mitotic figures, a good deal of variability is found, and in spite of the application of a method of random sampling, subjective judgment of the observer cannot be eliminated. Conclusions therefore must be tentative but the results suggest that the effects of ultraviolet light are mediated by some substance diffusing beyond the limits of the immediate photochemi-

cal effects of the radiation. Studies of carcinogenesis in albino mice with ultraviolet wavelengths that penetrate little below the epidermis have resulted in the induction of epidermal tumors only. This agrees with Dr. Blum's earlier studies to indicate that the carcinogenic effect of ultraviolet light is confined to the immediate site of action of the radiation, in apparent contrast to the case of transient hyperplasia. Interest has been maintained in the hazards of cancer of human skin from ultraviolet light, particularly that of sunlight, and the bearing of these experimental studies thereupon.

New experiments by Dr. White and his associates to determine the effect of tumor protein as a source of dietary nitrogen on the "protein reserves" of normal and tumor-bearing rats are now under investigation. Sprague-Dawley rats ingesting a low casein (6%) diet develop severe cirrhosis of the liver between 87-150 days of feeding. When fed 8% casein diet, cirrhosis was less severe. Even after 145 days many of the animals showed only moderate cirrhosis. Osborne-Mendel rats on 6% casein diet showed a much less severe cirrhosis up to 262 days; but on an 8% diet no cirrhosis was observed up to 210 days. At the end of 365 days, 15 of the 22 animals had cirrhotic lesions. Animals showing moderate to severe cirrhosis showed no reduction of these lesions when transferred to an 18% casein diet, indicating the damage was not reversible. Studies using tumor protein as a source of nitrogen are being compared with the above results. Attempts will be made to determine whether the cirrhotic effect can be reversed by other agents such as choline.

The office of this Laboratory has performed in a superior manner. In addition to the smooth administrative operation of the Laboratory, they have met deadlines, crash programs, last-minute manuscripts and the like, with equally superior fashion. All members have willingly worked long hours, without request for overtime or compensatory leave. Financial records have been kept on a weekly basis, making available to the Chief current expenditures and those to be encumbered.

The machine, electronics and glass shops of this Laboratory have made endless experiments

possible by design, engineering and fabrication. They have modified and repaired existing equipment which has saved many experiments and much valuable time. These shops have been available to other laboratories in the NCI and here too they have rendered valuable assistance.

It is indeed heart-warming to know that the administrative and shop personnel of this Laboratory have responded so enthusiastically and efficiently with their services.

Cancer Physiology Section

Dr. S. H. Wollman and research associates Drs. G. Andros and J. E. Loewenstein continue to study the mechanism of the iodide concentrating mechanism of the thyroid gland. They have been exploring some aspects of intrathyroidal heterogeneity which may help explain kinetics of radioiodine metabolism in the whole gland. Studies involving a peculiar type of cystic follicle in the thyroid of the C₃H mouse showed that although it did not accumulate organic iodine it could concentrate inorganic radioiodide to about the same extent as normal follicles. This cystic follicle also was responsive to TSH (thyroid stimulating hormone) stimulation in ways similar to the normal follicle. They have also studied intrafollicular heterogeneity, especially of the colloid, and have explored the effect of factors such as dietary iodine intake which influence the rate of diffusion of iodoproteins in the lumen of the follicle.

Dr. Rabinovitz and research associates Drs. Honig and Waxman are studying in both normal and tumor cells the path that amino acids follow leading to the synthesis of proteins and the controls exercised by the cells in regulating these processes. With Dr. Honig, Dr. Rabinovitz has been studying the inhibition of protein synthesis in Sarcoma 37 ascites cells by actinomycin D. Incubation of S37 cells in the presence of antinomycin D using pyruvate as a source of oxidizable substrate but lacking in glucose, leads to a marked inhibition of protein synthesis after 90 minutes. Cytoplasmic nuclearproteins were equally inhibited. This inhibition could be prevented and relieved by the addition of glucose. These data indicate that actinomycin D is not acting by reducing the

availability of messenger RNA directly required for protein synthesis, but is probably interfering with some ribonucleic acid required for utilization of amino acids under respiratory conditions. Similar observations were made with human chronic myelogenous leukemic cells. With Dr. Waxman he has been studying the role of iron and heme in maintenance of polyribosome structure and regulation of globin synthesis of rabbit reticulocytes. It is now generally accepted that the ribosomal aggregates (polyribosomes) are the sites of globin synthesis in reticulocytes as well as of a variety of proteins in other cell types. They have demonstrated that polyribosomes of reticulocytes can be disaggregated under conditions which bring about iron depletion, such as preincubation with amino acids to remove available iron by hemoglobin synthesis by binding the iron in cells with chelating agents. Polyribosomes do not disaggregate in cells when incubated with low concentrations of ferrous iron or hemin and under appropriate conditions disaggregated polyribosomes can be reassociated from ribosomes. Optimal hemoglobin synthesis is only seen in cells having high polyribosome levels and it is through the maintenance of the active aggregated state of ribosomes that heme supports the synthesis of globin. The mechanism at the molecular level for this cytoplasmic regulation of protein synthesis by a nonprotein component, heme, is not presently understood nor is it readily explained by the currently accepted theory of the mechanism of polyribosome action.

Dr. Reid continues his studies on the urinary excretion patterns in leukemia. This study requires the employment of chemical and mathematical techniques. The chemical procedures comprise an integrated chromatographic system developed by Dr. Reid which separates a urinary specimen into a large group of mixed fractions. The mathematical techniques which are primarily those of linear algebra represent an approach to further resolution of these mixed fractions by means of computational analysis of their ultraviolet absorption spectrum. The large volume of data thus generated is handled by a data processing machine. This approach to studying purine and pyrimidine excretion products in leukemia is extremely time

consuming and fraught with ever-rising problems. Although publications from this type of approach are not frequent, it requires one with the patience, knowledge of chemical, mathematical, and electronic procedures which Dr. Reid possesses to bring it to a successful conclusion. Several urinary components have been isolated which appear to be identical with pseudouridine itself except it gives no orcinol response and on electrophoresis and thin layer chromatography exhibits evidence of complex formation. It is suggestive of some isomer of pseudouridine. Work is continuing on the urinary profiles of different leukemic subjects but ultimate identification of peaks is needed before patterns can be established.

Energy Metabolism Section

The investigations on the total energy expenditure of the tumor-bearing rat as it is derived from both the host and the tumor have been continued by Drs. Morrison, Millar and Pratt. Present investigations are concerned with the patterns of heat production with concomitant recording of feeding behavior and other activity to better understand the mode and extent to which feeding behavior influences heat production and the impact that the presence of a tumor in various stages of growth has on the energy exchange. Recent observations indicate that in the normal rat there appears to be no distinction in terms of energy expenditure or activity pattern between the energy expenditure resulting from feeding activity and that resulting from non-feeding activity. Thus it appears likely that all non-resting energy expenditure will be equally affected by the potential of dissipating heat.

The energy metabolism pattern of normal rats is now being studied at various environmental temperatures. These experiments will determine whether the partition of total expenditure into rest, feeding activity and non-feeding activity is also altered. This may be relevant to the depression of food intake of the tumor-bearing animal which partially recovers its food intake on exposure to low environmental temperatures. Experiments are in progress on the metabolic pattern of rats after destructive lesions of the lateral hypothalamus, particularly on the relative changes in water electrolyte exchange

and nitrogen in tissue experiments both in the normal and tumor-bearing animals. Experiments on the etiology of the development of gastric ulcers in the rat during tumor growth have thus far indicated that the incidence of glandular ulcers in the presence of a transplanted tumor is a function of the condition of the carcass as influenced by a combination of circumstances (diet, tumor size and time) whereas ulcers of the forestomach are possibly related to stomach contents.

Dr. Pratt and Mr. William White have made rapid and important progress in developing mathematical and computer programming techniques which may be applicable to the investigator in the biomedical field involving information storage and retrieval problems. In collaboration with Dr. L. Thomas of the Laboratory of Pathology, a complete computer-based system has been devised for storing and retrieving of surgical pathology, autopsy pathology and cytology records. This affords the staff a rapid and precise identification of study material and provides a complete description of disease pathology. Similar programming systems have been devised for NCI and NIH grants. The former is in progress and formulation and computer programming of such grants should soon be completed. The latter consisted of a complete system analysis of the Division of Research Grants relative to the storage and retrieval of scientific content of the NIH Research Grants. This vast undertaking was completed in a relatively short period of time. Dr. Pratt in conjunction with Mrs. J. N. Toal has continued their mathematical and computational dealing with the ultraviolet spectrometric analysis of oligonucleotides. Previous reports of this successful approach for the analysis of the base composition of oligoribonucleotides have resulted in new experiments to apply this technique to determine nucleotide sequence simultaneously. Insufficient work has been done to determine if the computational approach will be a useful adjunct, laboratory procedure.

Physical Biology Section

The characterization and comparison of the nucleic acids and nucleoproteins of normal and malignant tissue are under investigation by Dr.

Shack. A good part of the time has been spent on methodology involving centrifugal and electrophoretic procedures so that quantitative isolation and subsequent fractionation could be carried out with microgram rather than milligram amounts of DNA. This has been achieved through the isotopic labeling of DNA. It has now been possible to separate a trace of double stranded DNA from a large amount of single stranded DNA and a trace of single stranded DNA from a large excess of double stranded DNA. This procedure of separation was of particular significance in the attempt to separate and concentrate certain species of DNA such as viral DNA. Work is in progress to attempt to determine whether DNA of viral origin is present in tumors caused by viruses.

Dr. Breitman is continuing his work on the control of ribo- and deoxyribonucleotide synthesis. Studies on the metabolism of two thymineless strains derived from *E. coli* have continued. He plans to develop an assay for ribonucleotide reductase activity, the enzyme system which synthesizes deoxynucleotides from ribonucleotide precursors; to elucidate the mechanism of thymine to thymidine conversion; and to establish the exact action of thymidine on ribonucleotide synthesis.

Radiation Biology Section

Studies on the repair of sublethal damage in X-irradiated mammalian cells by Dr. Elkind have been extended. He has shown: (a) That the repair of damage is weakly dependent on temperature; some repair is evident even at 1°C. (b) Repair of damage is not an oxidative process; repair proceeds quite well under the simultaneous conditions of hypoxia and suboptimal growth temperatures. And (c), the gross effects of irradiation on macromolecular synthesis show that the pattern of DNA synthesis is strongly affected. The patterns of RNA and protein synthesis are weakly affected. In contrast, the RNA inhibitor, actinomycin D, has a strong effect on the repair process while DNA (5-fluorodeoxyuridine) and protein (puromycin) inhibitors have minor effects.

The quantitative studies of radiation damage, spontaneous recovery and induced recovery on the hemopoietic system are actively being pursued by Dr. W. W. Smith. More recently

she has found that if endotoxin is given before or shortly after irradiation, the number of colony-forming units arising increases endogenously in the spleen. Endotoxin given to nonirradiated animals increases the number of colony-forming units in the spleen (determined by injection into irradiated recipients). Endotoxin causes a relatively early return to normal in both spleen and bone marrow forming units. Experiments are underway to determine whether or not the numbers drop as low in treated as in untreated animals. Kinetics of the recovery process in animals treated after irradiation are underway.

Dr. Draper is pursuing his studies on antibody formation following multiple irradiation and immunization procedures. The goal of this research is to determine the sources of natural and specific hemolysin during the recovery from irradiation damage and whether there is a selection site of production of antibody type following irradiation exposure. A modification of a method for the determination of the avidity of hemolysis produced in rabbits has been made which permits the use of the same preparation of untreated cells for both cell populations used in the system and thereby eliminates the possibility of differences between them with respect to their affinity for the hemolysin or their susceptibility to spontaneous or immune lysis. The relevance of this parameter, that is hemolysin transfer to avidity, has been verified by dilution effect experiments in which the avidity of antibody is measured in terms of the persistence of antigen-antibody complexes upon the dilution of the reaction mixture. The less avid the antibody, the higher its rate of dissociation from antigen: dilution reduces the probability of reassociation. Thus the reduction in the number or proportion of complexes is a function of the dissociation rate of the antibody. The dilution effect is minimal with avid hemolytic antibody showing little or no tendency to transfer and vice versa.

Dr. Riesz has recently been concerned with the effects of ionizing radiation on biologically active macromolecules. In collaboration with Dr. F. H. White, NHI, they have been determining the effect of radiolysis on dry ribonuclease. They have devised a new method for studying the distribution of free radicals in irradiated

macromolecules *in vacuo*. Stable free radicals are produced and reacted with tritiated hydrogen sulfide. Employing electron spin resonance measurements, about two-thirds of the carbon radicals are removed in less than 20 minutes at room temperature and the remainder can be removed by heating to 70°C. Thus the distribution of carbon-bound tritium among the amino acid residues is presumably a good approximation of the original radical distribution produced as indicated above. Following the removal of exchangeable tritium bound to oxygen, nitrogen and sulfur, the distribution of carbon-bound tritium among the amino acid residues can be determined. The carbon-bound tritium incorporated into amino acids does not correlate with the number of primary, secondary or tertiary carbon-hydrogen bonds in a given amino acid residue and free radical formation proceeds in a *non-random* fashion. It seems likely therefore that the distribution of free radicals depends on the conformation of the polypeptide chain.

Dr. Charles R. Maxwell has undertaken with Dr. Elizabeth S. Maxwell, NIAMD, a study of the effect of ionizing radiation on the cell to determine at what site the damage occurs and its effect on normal function. These studies will entail work on the ribosome, transfer RNA and protein synthesis. This initial work will be done on the rat liver homogenate.

LABORATORY OF CHEMICAL PHARMACOLOGY

The research work in the Laboratory of Chemical Pharmacology continues to stress physiological disposition and distribution of anti-neoplastic agents.

Folic acid antagonists represent one important class of chemotherapeutic compounds. A number of pteridine precursors have been successfully synthesized and 3'-idoaminopterin has been prepared. This compound, while not importantly active in L1210, could be of use in tracer studies if an iodine radioactive isotope can be substituted. The studies of the physiological disposition of methotrexate in animals and man reported last year have been continued. More animals and more patients have been added. No unexpected results have been obtained. The observation that oral administra-

tion of large doses of methotrexate results in slow and incomplete absorption, has been amply confirmed. This pharmacological observation is important in view of the observation by the Leukemia B Group that intermittent oral methotrexate may be as effective as intermittent parenteral methotrexate in childhood leukemia. Perhaps the long continued absorption of a smaller amount of methotrexate is as toxic and as therapeutically active as the rapid absorption of a larger amount of methotrexate. It is interesting to note that in plasma levels in man after 100-fold, different i.v. doses are parallel and have the same volume distribution. Studies on the plasma protein binding of methotrexate have been performed. Approximately 50% of the plasma concentration of methotrexate is bound to the plasma proteins, largely albumin, in mouse, rat, dog, and man. Of particular interest is that a number of weak organic acids, including the salicylates, sulfonamides, and probenecid, can displace methotrexate from plasma protein. This can result in increased lethal toxicity when a long acting sulfonamide, sulfamethoxypyrimidine, and methotrexate are administered simultaneously. Studies of the displacement of dichloromethotrexate have not yet been performed. Since this agent is 90-95% bound to plasma protein, displacement could have serious toxicological effects. In fact, the possibility exists that some of the erratic toxicity reported after dichloromethotrexate administration in man might be a result of concomitant administration of aspirin, sulfonamides, penicillin, and so forth. These studies will be followed with great interest. Basic physico-chemical studies of the relationship to protein binding and the solubility of a variety of relatively insoluble organic amines has been performed. It is interesting to note that the protein binding and aqueous solubility of these compounds correlates well. This suggests that the solvation of the protein and the chemical might be related to the mechanism of reversible protein binding.

The use of methotrexate in ventriculocisternal or ventriculoventricular perfusions for meningeal leukemia or intracranial neoplasms has been extended this year. Nine patients have been studied with 30 or more perfusions. A maximum dose of 75 micrograms per milli-

liter of methotrexate in 500–1000 milliliters has been used. No overt toxicity has been seen and, in the patients who have come to autopsy, no histologic evidence of toxic effects of methotrexate have been seen. The solid tumors have not been of such a type that therapeutic evaluation has been possible. However, in the two patients with meningeal leukemia, complete remission of the meningeal leukemia was obtained. In one patient, who died some months after perfusion, no leukemic cells were found at autopsy in the brain.

In an interesting study total body pyrimidine utilization was studied in chronic leukemic patients given 6-azauridine. The surprising finding was that 6-azauridine increased pyrimidine utilization during treatment. Perhaps this is a reflection of the inactivity of 6-azauridine against human neoplastic processes.

The metabolism of hydroxyurea has been extensively studied. A significant proportion of the hydroxyurea is converted to urea but interestingly enough 10% is excreted as carbon dioxide. The mechanism of transformation of hydroxyurea to carbon dioxide is not as yet understood.

Preliminary studies have been performed with cytosine arabinoside. The one significant finding has been the demonstration that an abnormal cytosine nucleoside was a contaminant of a commercially produced cytidylic acid. This developed out of a study of cytidylic acid concerning its ability to reverse cytosine arabinoside's antitumor activity. This "cytidylic acid" was found to have antitumor activity itself. The toxicity of cytosine arabinoside in dogs and monkeys is consistent with the human observations that the major effects are on the granulocytes. Otherwise the toxicity was not remarkable.

Methyl isopropylcarbamyl benzyl hydrazine (MIH) has been studied with respect to its biochemical degradation in the body and its distribution. The degradative pathway seems clearly to involve an azo compound and methylhydrazine. Considerable amounts of p-isopropylterephthaic acid are excreted as well as a variety of other small molecular weight related compounds. Certain intermediates may inhibit monamine oxidase. Studies on the carcinogenicity of MIH have been shown to occur in a

number of species of mice. C57 black strains are relatively resistant. In rats, MIH causes mammary tumors as well as a great variety of adenomas and carcinomas. The variety of tumors seen in some of the rats given MIH is reminiscent of the tumors seen in hamsters given polyoma virus. Studies of the carcinogenesis of MIH in the rhesus monkey are under way.

Two methods to determine the physiological disposition of bis- β -chloroethylnitrosourea (BCNU) have been employed this year. One method, a chemical method, has shown that the unaltered molecule rapidly entered the cerebrospinal fluid. It disappeared from blood and spinal fluid quite rapidly, and only traces were found in the urine. Studies using radiolabelled BCNU (C^{14} in all four chloroethyl carbons) have shown that in the mouse, while most radioactivity appeared in the urine, a significant amount was in respiratory CO_2 and small but significant amounts remained in liver and kidney. In the monkey, considerable amounts of radioactivity remained in various body tissues after 24 hours. It is notable that measurable plasma levels of radioactivity were found as long as 72 hours after a single dose of BCNU. It is clear, of course, that this does not persist as unaltered BCNU. It will be interesting to determine what these degradation products are. The ultrastructure changes following BCNU in liver, kidney, lung, and intestine of rats treated with this agent have been investigated. Perhaps the most significant observation which might relate to the delayed toxicity of BCNU is the profound effect seen on the blood vessels of the involved organs. This is manifest by a thickening of the walls and obliteration of the small vessels. Other important effects seen were desquamation of the renal tubules, unexplained dense inclusion bodies in the cytoplasm of hepatic cells and intestinal mucosa cells. A variety of other nitrosoureas have been studied. The other agents so far studied are fraught with problems related to their physical characteristics such as low water solubility and instability. Thus far no agent seems superior to BCNU. One interesting observation is that BCNU in cottonseed oil appears to be stable for long periods of time. The possibility exists that this might be a useful means of administering intramuscular BCNU.

We also studied some of the properties of dimethylsulfoxide (DMSO) this year, although these studies were not reported in *Life*, *Time*, or *Newsweek*. DMSO is an agent which has had many spectacular attributes claimed, including a variety of pharmacological actions in addition to that of being an excellent solvent. One of the claims was that it enhanced the permeation of compounds through cellular membranes. If this could be substantiated, it would be a great help in the problem of attacking the pharmacological hideouts of tumor cells. We studied the ability of DMSO to enhance the cellular penetration of a variety of pharmacologic agents. These included the entry of para-aminohippurate into the spinal fluid and in brain as well as into other tissues and the oral absorption of morphine, insulin, penicillin, and a number of other drugs. These studies were uniformly negative. No difference in concentration ratios or in intensity or duration of pharmacological action could be found when the compound was dissolved in DMSO as opposed to solution in water alone. Interest in this area leads us to study tetramethylurea, another agent which might be a useful solvent. Tetramethylurea does have excellent solvent properties. However, its toxicity, though not great, is significantly greater than DMSO. Interestingly it has a small amount of anti-tumor activity in certain experimental mouse tumors.

Studies on the mechanism of action of nitrogen mustard on DNA have been continued. The finding reported last year that the sensitive *E. coli* cells, i.e. sensitive to nitrogen mustard, exhibit a lack of ability to repair the crosslinks caused by the nitrogen mustard. This year's studies have been directed more towards the kinetic of reaction between nitrogen mustard and double stranded DNA. These kinetics seem to be essentially first order after a pronounced lag. These observations are explained by the notion that one crosslink will denature DNA. The lag is a function of the time it takes to make the first reaction between the chloroethyl group and the base and the first order kinetics then follows as the second joins the opposite base. These studies are interesting in their use of a variety of sophisticated techniques including the IBM 620 computer to validate the mathematical models.

Of increasing importance, it seems to us, will be the use of human and animal cell lines grown *in vitro*. By the use of a combination of DMSO and DNase, freezing and recovery of the Grace human myelogenous leukemia cell line and a variety of other cell lines has been shown to be feasible. If attention is paid to the details, this is a practical and simple method. An important development has been the perfection of a method of synchronization of mitotic events in tissue culture. This method utilizes the changing adhesiveness of cells during the mitotic cycle to extract a population of cells homogeneous with respect to their mitotic cycles.

The development of hepatic carcinomas in five monkeys given dimethylnitrosamine has not only been of interest in terms of questions of susceptibility of primates to carcinogenesis, but provides a new experimental tool in which studies on hepatomas may be performed. In collaboration with the Surgery Branch and the Diagnostic Radiation Branch, angiograms have been used to demonstrate the extent of the hepatomas in certain of these animals. In addition the possibilities of direct cannulation of hepatic vessels and the treatment of these hepatomas with a variety of appropriate anti-neoplastic agents is being actively pursued.

Combination toxicity studies of anticancer agents are well under way. No specific information of any startling variety is yet available. The difficulties of designing and interpreting these experiments are slowly being overcome. The importance of combining agents with independent action is becoming more apparent. An urgent need is seen to adapt the Skipper cell kill model to the P1798 tumor which is susceptible to all of the clinically useful leukemic agents and then to use this system in combination experiments to allow for the estimation of therapeutic effectiveness and host toxicity in parallel closely related experiments.

Studies designed to elicit distribution of anti-cancer drugs in a variety of potential pharmacological hideouts such as the thymus gland, the center of necrotic tumors, and so forth awaits the installation of the ultra-microtome which will allow whole animal autoradiography of medium sized rats.

An important activity of the chief of this laboratory (with substantial assistance from a

variety of staff members) has been participation in a committee organized by the Director of the National Institutes of Health to consider overall problems of the state of the art of the pharmacology today and to determine whether strenuous efforts should be made by the NIH to enhance the effectiveness of this important discipline. This has resulted in a scientific paper embodying certain important recommendations which will appear in Science in the near future. Out of this, I think, has come an awareness that the important concepts in pharmacology are related to the interaction between chemical substances and biological material and have many implications in most aspects of biomedical research. Implicit in this is the awareness that one must look beyond the intimate effect of a chemical agent on some specific and discrete biological or biochemical system and be aware of potential implications for not only the organ system but the whole animal, clinical therapeutics, medical practice, and public health in general.

MEDICINE BRANCH

The 1964-65 fiscal year has been a very eventful one for the Medicine Branch of the NCI in many respects. A major administrative change occurred when Dr. Emil Frei resigned to accept an important position at the University of Texas M.D. Anderson Hospital in Houston, Texas. Dr. Paul Carbone was appointed the Head of the Solid Tumor Service, a service which was created last year when the clinical program was divided into 2 sections. The other section, the Leukemia Service, continues under the direction of Dr. Emil Freireich.

One of our senior investigators, Dr. Robert H. Levin was killed in an airplane accident. He was a young and bright investigator who had already made a number of important contributions in the clinical study of acute leukemia.

Excellent and close collaboration has been maintained between the Laboratory of Chemical Pharmacology and the Medicine Branch. This is one of the most important and mutually beneficial interactions in which the Medicine Branch participates.

In the past 12 months a number of problems, largely administrative, have arisen with the

Chemotherapy Tumor Group of the Baltimore U.S.P.H.S. Hospital which has prevented an effective collaborative effort. Without the availability of this facility it has not been possible for the Medicine Branch to perform the number of Phase I and II chemotherapy studies which are necessary in a major clinical anti-tumor therapy program. Hopefully these problems will be resolved in the near future.

Activities in the Solid Tumor Service have gradually become disease-oriented during the past year with emphasis on the lymphomas particularly Hodgkin's Disease and on the testicular tumors. This is in addition to the interest in intensive chemotherapy of the blastic crisis in chronic myelocytic leukemia.

Lymphomas

Several important findings have emerged in patients with lymphomas including Hodgkin's Disease. An intensive combined drug and radiation study in 14 patients with Hodgkin's Disease was completed. They were treated with a combination of vincristine, cyclophosphamide, methotrexate, and prednisone given in two week courses and combined with radiotherapy in patients with Stage I or II disease. Moderate reversible toxicity occurred in all patients but tumor regression was attained in 13 of the 14 patients with chemotherapy alone prior to radiotherapy. Treatment was not successful in 4 of the patients—all in Stage III (out of 10 in that category). Most of the patients are still being followed but a significant finding to date is that in the responsive patients with Stage III Hodgkin's Disease the median duration of unmaintained remission is approaching 10 months. In the other stages, the median of unmaintained remission is over 12 months. This is considerably better than with any other known drug. This study is continuing with a slightly different combination of drugs but with the same objectives in mind. In the new study patients with the other lymphomas are also included.

A large cooperative study of lymphomas with the Eastern Solid Tumor Group and the Acute Leukemia B Group is being conducted under the co-chairmanship of the NCI. In this study delayed remission induction and duration of remission both maintained and unmain-

tained have been determined. The study has established that a vinca alkaloid is as effective as an alkylating agent in the treatment of patients with lymphosarcoma and Hodgkin's Disease.

Combination Therapy in Solid Tumors

In patients with solid tumors, a study has been completed in which a combination of drugs was used to determine the relative rate of remission as well as toxicity. Cyclophosphamide, oncovin, methotrexate, and 5-fluorouracil were given at almost full dosage for 5 day periods with a rest period of 14 days between courses. This combination was well tolerated and toxicity was not excessive. There was a significant incidence of tumor responses in patients with carcinoma of the breast and with testicular tumors.

This study is being continued in an attempt to determine the relative value of the concurrent use of antibiotics on hematologic and gastrointestinal toxicities. One group receives oral non-absorbable antibiotics and the second group acts as a control. Patients are randomized in the usual fashion. To date; 20 patients have been studied in this program with 11 having received antibiotic therapy and 9 in the control group. Initial data suggest that the duration of fever and the incidence of infection in the treated group are somewhat lower but the differences are not statistically significant at this point.

Chronic Myelocytic Leukemia

Intensive studies in patients with chronic myelocytic leukemia continue. These encompass all aspects of the disease. About 85% of the patients with this clinical diagnosis will have the Philadelphia chromosome. It also appears that those who do not have the abnormal chromosome have a shorter survival, that is, 18 months compared to 45 months in the individuals who are Ph¹ positive.

A major effort is being made to control the blastic crisis of patients with chronic myelocytic leukemia. Combination drug treatment with prednisone, methotrexate, and 6-mercaptopurine (PAP) has been used. Three of five patients who received one course of drug treat-

ment have gone into remission. However, these remissions have been of very short duration lasting from 1 week to 3 months. Treatment in this stage of chronic myelocytic leukemia is made more difficult because it is not possible to make a definitive diagnosis early. Hence, the possibility remains that intensive therapy is being started too late in the course of the complication and that success would be more likely if therapy were begun at an earlier time. Leukocyte kinetic studies with tritiated thymidine may be of value in arriving at an earlier diagnosis.

Acute Leukemia

The major advance in chemotherapy in acute leukemia during the last year was the development of what appears to be effective chemotherapy for adult leukemia. Remission induction was achieved in 19 of 24 patients with acute myelocytic leukemia over the age of 20. This was accomplished with the POMP combination chemotherapy program (prednisone, oncovin, methotrexate, 6-mercaptopurine).

In children with acute lymphocytic leukemia it has been shown that intensive therapy early in remission can significantly prolong the duration of unmaintained remission. In addition, better techniques for maintaining remission once achieved have been evolved using intermittent methotrexate therapy. A study of the importance of the duration of treatment of patients in remission should permit the design of remission maintenance therapy which will very greatly prolong the duration of the initial remission.

Multiple Myeloma

A study has been completed using Cyclophosphamide in multiple myeloma. It appears to be as effective as phenylalanine mustard. Methotrexate and 6-thioguanine have also been tried but were found to be ineffective in this disease.

A cooperative effort with the Metabolism Branch and the NIAID and NIDR has been completed in which fluoride was utilized in the treatment of bone disease in patients with multiple myeloma. It appears that fluoride will correct the negative calcium balance in these patients with incorporation of the halogen into

bone as determined by chemical analysis of biopsy specimens. Studies of the crystalline structure of the bone as well as its histology are now underway.

New Agents

During the year several new agents have undergone initial evaluation. The most promising of these has been methyl isopropyl carbamyl benzyl hydrazine (M.I.H.). This drug shows no cross resistance to any known chemotherapeutic agent and has induced a 50-75% remission rate in Hodgkin's Disease.

The effect of cytosine arabinoside in patients with multiple myeloma is being evaluated. This drug has also been tested in a relatively small number of patients with chronic myelocytic leukemia but does not appear to be particularly promising at this time. Early studies in acute leukemia suggest that it may have some value both in remission induction and maintenance.

Leukocyte Dynamics in Leukemia

Studies of the leukocyte kinetics in patients with leukemia, primarily chronic myelocytic leukemia continue. Although the analysis is not yet complete it appears that in these patients, in contrast to normal individuals, the cells are synthesizing DNA in the bone marrow as well as in the peripheral blood and that the cells are released from the bone marrow and/or spleen into the peripheral blood. These cells then re-enter the marrow and spleen. Cell division in the bone marrow appears to progress only to a certain point and does not appear to continue in the peripheral blood. The myelocyte is the predominant cell involved. If any significant number of this large group of cells in this disease were to divide, many labelled mature granulocytes would appear in the peripheral blood at some time during the period of observation. However, this does not seem to be the case as determined from the analysis of the radioautographs. The studies have also suggested that cells in chronic myelocytic leukemia survive or recirculate in the peripheral blood with a half-time between 60 to 90 hours. Similar studies in patients with acute myelocytic leukemia have suggested that the intravascular survival (half-time) is approximately 12 hours. Of

particular interest is the observation that patients in the blastic crisis of chronic myelocytic leukemia have curves which appear to be identical with those of patients with acute leukemia and in marked contrast to the curves seen in patients with chronic myelocytic leukemia. This observation may be important in the diagnosis of this particular phase of the disease.

Leukocyte and Platelet Transfusion

The white blood cell and platelet transfusion program continues to constitute a major effort in the Medicine Branch. The availability of a plasmapheresis unit in the acute leukemia area has become a very valuable resource for the collection of leukocytes, the improvement of platelet concentrates, and for the collection of plasma from patients.

A more effective technique has been devised for the preparation of platelet concentrates in acidified plasma which has virtually overcome the problem of preparation of good platelet concentrates. This new technique results in a preparation of platelet concentrates which are equal in efficacy to the platelet-rich plasma preparations.

Granulocyte transfusions as a supportive measure are being used with increasing frequency. Previous studies with transfusions of granulocytes obtained from patients with chronic myelocytic leukemia have suggested that this type of replacement therapy may be valuable as a supportive measure during the marked leukopenic stage resulting from intensive chemotherapy. However, during the past year in addition to these studies, high dose normal white cell transfusions have been initiated using Dextran separated granulocytes from freshly drawn units of normal blood. Preliminary results suggest that the percent recovery normal granulocytes is similar to that obtained with the leukemic granulocytes. Effectiveness of homologous normal granulocytes in controlling infection in leukopenic individuals is difficult to evaluate primarily because of the problems in obtaining sufficient numbers of normal cells. As soon as these become available in quantity, it will be important to perform a comparative study with leukemic granulocytes to establish relative effectiveness.

It had been hoped that with the development of a continuous white blood cell separator that there would be less reliance on the cells from patients with chronic myelocytic leukemia for transfusion purposes. However, although the separation of leukocytes from bank blood has been quite satisfactory, the yield on an *in vivo* basis has been poor. Intensive study of the reasons for this variance between fresh blood and bank blood has revealed that the difference is probably due to a greater tendency toward rouleaux formation in stored blood. Additional studies indicate that the densities of leukocytes and erythrocytes overlap. Accordingly, it has become quite obvious that the separation of these two types of cells will be feasible only by the use of a medically acceptable additive. This study is now proceeding and the early results appear to be promising.

In the course of the development of the leukocyte separator, other uses for the machine have suggested themselves. With relatively minor modification of the present design it is now possible to procure large numbers of platelets and lymphocytes. Separation of the plasma and the cellular elements of blood *in vivo* on a continuous basis as well as *in vitro* is quite practical. Finally, a modified version of the present machine can be used for the deglycerolization of frozen blood in less time than by any other known method.

"Life Island"

A development of potential major importance during the last year appears to be the introduction of the "Life Island" reverse isolation technique. Four patients have been entered but only three patients can be evaluated including one individual with acute lymphocytic leukemia, one with acute myelocytic leukemia, and one with a testicular carcinoma and widespread metastases. All received amounts of chemotherapy which under ordinary circumstances might have resulted in life threatening toxicity. However, in the "Life Island" toxicity appeared to be relatively moderate. Leukopenia was severe in all three individuals but there were no infections, buccal ulcerations, or other toxic manifestations. Response to therapy was quite satisfactory in the first two patients. The

third patient, who is still in the unit at this time, has shown complete tumor regression. These preliminary studies suggest that it may now be possible to administer chemotherapy at dosages far in excess of what is ordinarily given at a risk of comparatively moderate toxicity.

Biochemistry of Leukemic Leukocytes and Malignant Cells

The biochemistry of leukemic leukocytes and malignant cells in general continues to be an area of increasing emphasis within the Medicine Branch.

It has been suggested by a number of individuals that thymidine may exert a controlling or regulatory influence on DNA synthesis in leukemic cells. Accordingly, the effect of thymidine on nucleic acid and protein synthesis in the leukocytes from patients with chronic and acute leukemia has been studied intensively during the past year. It appears that the inhibition of DNA synthesis is dependent on the constant presence of thymidine in the medium but is reversed by its removal or by the addition of deoxycytidine. The importance of pre-formed or exogenous thymidine in DNA synthesis in leukemic cells has not yet been clearly established. However these studies have indicated that low concentrations of thymidine inhibit DNA synthesis. It is intriguing to speculate on this since thymidine in significant quantities is readily available from dietary sources as well as from endogenous pools by catabolism of DNA released from disintegrating cells.

Further studies with other inhibitory substances which have been isolated from leukemic white cells continue. Although the evidence is not yet definitive it may be that thymidine phosphorylase, described last year, may be the inhibitory substance responsible for the indirect effects of splenic irradiation, remissions obtained in leukemia by hypertransfusion, etc.

In addition to studies of DNA synthesis in human leukemic cells, RNA synthesis in lymphocytes of patients with chronic lymphocytic leukemia has been investigated. These cells are capable of synthesizing large amounts of relatively stable RNA. Using the technique of DNA-RNA hybridization one can demonstrate

that one-third of the RNA is rapidly synthesized and that it can hybridize with homologous DNA. In addition, a large portion of this RNA resembles ribosomal RNA. These components are present even after 24 hours of incubation, a situation quite different from other mammalian cell lines.

Virus Studies

In close collaboration with the Laboratory of Viral Oncology the isolation and identification of the virus-like particles found in patients with leukemia is being pursued. In addition, the Medicine Branch is continuing to assist the Laboratory of Viral Oncology in the immunological study of the relationship between these virus-like particles and the presence of leukemia.

A finding of potentially great importance but is still not definitive is the observation that about $\frac{1}{3}$ of the patients with leukemia had particles in their urine on electron microscopy which resembled those seen in plasma of the patients with leukemia by Dalton and his collaborators.

Conclusion

It is apparent from the foregoing review of the activities of the Medicine Branch that senior members of the branch have become heavily involved in research activities which are vital to a comprehensive study and understanding of the etiology, mechanisms, and treatment of leukemia and solid tumors in man. These include: 1) studies of immune competence in malignant disease and the effect of drugs, 2) cytogenetics, 3) physiology and dynamics in leukemia, 4) biochemistry of leukemic leukocytes and malignant cells, 5) platelet and leukocyte antibodies, 6) bone marrow transplantation, (a) in support of patients with malignancies who have undergone intensive treatment, (b) in leukemia after "ablation" therapy, 7) in collaboration with other groups, studies of the incidence of virus-like particles, PPLO, and cytomegalic inclusion virus in leukemia, 8) perfusion studies for (a) bone protection and (b) for the investigation of tumor blood flow for the possibility of enhancing drug exposure.

With all these areas under investigation it might be of value from an administrative standpoint to consider a creation of additional sections to include cytology—cytogenetics, biochemistry (leukemia, virus) and perfusion. At least 2 additional senior individuals are needed—a research cytogeneticist and a biochemist with a predominant interest in leukocyte biochemistry, control mechanisms, and viral effects. These would fill voids which now exist.

Having these tremendous resources, the Medicine Branch is in a highly favorable position to make major contributions in leukemia and certain of the malignancies. It appears that of all the malignancies, leukemia should be the first disease amenable to a solution and cure. This disease(s) is being approached from all angles—etiological, physiologic, biochemical and therapeutic. In the solid tumors, it has become obvious that greater emphasis should now be placed on "disease orientation" rather than on drug orientation. The clinical drug evaluation program has provided a number of drugs which singly or in combination result in significant tumor regression and remission rates in three diseases: 1) the lymphomas, particularly Hodgkin's Disease, 2) testicular tumors, 3) carcinoma of the breast. It is these, in my opinion, to which our attention should now be directed. With the very early promising results in the "Life Island" and with the support of blood products, "maximum" antitumor therapy may now be possible without prohibitive toxicity.

LABORATORIES OF VIRAL CARCINOGENESIS AND VIRAL ONCOLOGY

During the past fiscal year activity was increased in the tumor-virus program of NCI and the large Laboratory of Viral Oncology was split into two laboratories under the newly created office of Associate Scientific Director for Viral Oncology. Dr. Bryan was appointed Associate Scientific Director but also remained as Chief of the Laboratory of Viral Oncology. Dr. Dalton was appointed Chief of the second laboratory, designated as the Laboratory of Viral Carcinogenesis. Scientific members of both laboratories continue to work together as a

single functional group with respect to participation in the collaborative human cancer-virus program of the Institute, and, during the last half of the year, several of them accepted responsibility for the administration of segments of the newly created Special Virus-Cancer-Leukemia Program of the National Cancer Institute. Doctors Dalton, Fink, Manaker and Moloney are serving as Program Segment Chairmen or co-Chairmen, and Dr. Rauscher is the scientist member of the Management Team of the Special Program. Doctors P. Mora, T. O'Connor and S. Stewart are members of Segment Working Groups.

Within the two laboratories the spectrum of scientific disciplines and special skills ranges all the way from the molecular level to the level of the intact animal host, including man. Although emphasis on research is directed primarily toward viruses in relation to cancer, the research effort also includes studies on basic problems underlying solutions of tumor virus problems. These include: the interactions of macromolecules in isolated and interpretable *in vitro* systems; the ultrastructure of cells, including normal and non-viral cancer cells as well as cells of virus induced cancers; immunological phenomena in relation to cancer; basic virology, including viral ultrastructure and virus-host interactions *in vitro* and *in vivo*; and the development and refinement of technology within these areas.

In the Laboratory of Viral Carcinogenesis Dr. Sarah Stewart has succeeded in establishing a continuous line of malignant cells from a biopsy of Burkitt lymphoma in a 7-year-old Nigerian male who was admitted to the Clinical Center. In collaboration with Dr. Stewart the cells in tissue culture were shown by Dr. Dalton to carry a virus which in electron-microscopic morphology resembles the herpes viruses and the Lucké frog kidney carcinoma virus. The virus is morphologically the same as that found in 3 different established cell lines derived by Epstein (London) from as many different cases of Burkitt lymphoma. This brings to 4 the number of successfully established cell lines of this tumor, and each of the 4 lines carries the same type of viral agent. Studies by Epstein on his cell lines have shown the virus to differ from herpes virus in both immunologi-

cal and biological properties. The virus is now considered to be a serious candidate for an etiological agent of Burkitt lymphoma. Additional studies by Dr. Stewart succeeded in establishing conditions under which a viral agent from the Burkitt cell cultures, presumably the agent identified with the electron microscope, can be passaged serially in hamsters. This work is still in an early phase but if repeatable with the other 3 candidate virus isolates, this could provide the basis for rapid animal bioassays and thereby revolutionize the problem of determining etiological significance of the Burkitt-associated-virus. Other studies of Dr. Stewart aimed at finding susceptible hosts for possible agents recoverable from human leukemia patients led to the discovery of a new canine virus. Although "herpes-like" in electron microscopic structure, it differs from herpes as well as from previously known canine viruses. The virus is transmissible in suckling pups in which it produces lymphadenopathy and hemorrhagic lesions. There is evidence also that this agent is responsible for some cases of canine abortion. The electron microscopic studies were carried out by Dr. David-Ferreira, in collaboration with Dr. Stewart. Further investigations of the "factor" previously reported by Dr. Stewart which enabled standard strains of Rous sarcoma virus (a chicken virus) to cross species into mammals (hamsters) have indicated that it probably is a non-specific enhancing factor, and not a unique substance which endows a new qualitative, species-crossing property. This is further indicated by the finding of Dr. Rabotti that very high doses of all strains of sarcoma virus thus far tested are capable of inducing tumors in hamsters without any added enhancing factor. It should be pointed out that an intensive research effort (under contract) directed toward the isolation and identification of this "factor" led to the discovery that a known chemical substance, dimethylsulfoxide (introduced as one of the "controls"), also enhanced the biological activity of Rous sarcoma virus. The inclusion of dimethylsulfoxide in the suspending fluid was a crucial part of the procedure developed by Dr. Stewart for successful passage of the Burkitt-associated-virus in hamsters.

Dr. Manaker has succeeded in developing a continuous line of malignant cells from an adult male who died of acute lymphotic leukemia about one month after the expiration of his wife with the same disease. A report just received from the contract supported electron microscopy collaborative group at the Pfizer Company (Maywood, N.J.) indicates that this tissue culture cell line carries a "herpes-like" viral particle similar to that carried by the Burkitt lymphoma cells as well as by two cell lines derived from human myelocytic leukemia (Grace, Roswell Park). In studies on an avian tumor virus isolated from a methylcholanthrene-induced tumor of a Japanese quail (*Coturnix* sarcoma virus (CSV), by Reyniers, Tampa), Dr. Manaker has succeeded in propagating the agent in quail tissue cultures and has determined some of its properties. The virus is highly stable and appears not to be of the defective type as is the Rous sarcoma virus of chickens. The tissue culture-grown quail virus produces tumors in chickens which regress after about three weeks in this host. Further studies on biological and immunological properties are in progress. Studies on several established cell lines of mouse myeloepithelioma by Dr. Manaker in collaboration with Doctors Rabotti and David-Ferreira have shown that this mouse tumor carries immature "type C" virus particles, but biological tests have failed to show transmissibility of this neoplasm by filtrates. A small but significant portion of the inoculated mice develop leukemia, indicating that the tumor cells may be carriers of a mouse leukemia virus. Dr. Manaker is leading an intensive collaborative effort involving both intramural and extramural (contract supported) scientists of various disciplines in attempts to increase the production of Burkitt-associated-virus and isolate and purify it from lymphoma cells in tissue culture.

Dr. Rabotti has carried out quantitative studies on the induction of brain tumors in hamsters by Rous sarcoma virus (a chicken virus), and found that standard strains of this virus, previously thought incapable of crossing broad species barriers, can induce brain tumors in hamsters if the inoculated dose is high enough. The oncogenic dose of standard Rous sarcoma virus was thus found to be 10^6 PFU,

whereas other strains such as the Schmidt-Ruppin strain previously shown to cross species readily requires only about 10^3 PFU. Exploring this quantitative finding further and using very large doses of Rous sarcoma virus, Dr. Rabotti has succeeded in inducing brain tumors in guinea pigs, rabbits and dogs. The tumors in guinea pigs were leptomeningeal sarcomas, only, whereas in rabbits and dogs both gliomas and leptomeningeal sarcomas were induced. Both Schmidt-Ruppin and standard strains of the virus were effective provided "oncogenic doses" were used. This technique for rapidly inducing tumors in dogs (within a few weeks) is of considerable importance to cancer chemotherapy and related pharmacological investigations because, up to now, there has been no available experimental tumor of large animals for use in pharmacological studies, prior to trial of candidate therapeutic agents in man. The brain studies in dogs were in collaboration with Dr. A. Grove (NINDB). Dr. Rabotti is also collaborating with Dr. D. Rall, LCP, in developing the use of dog meningeal tumors as a test system for trials of chemotherapeutic agents selected for potential effectiveness in the treatment of meningeal leukemia.

Dr. Dalton and other electronmicroscopists of his group continue to provide the "eyes" in the search for viral agents associated with human neoplasia, particularly leukemia, as well as participating in multidisciplinary investigations on both human candidate tumor viruses and viruses of known animal viral neoplasias. As already mentioned, Dr. Dalton has studied cells of the Burkitt lymphoma line (SLI) established by Dr. Stewart and has observed "herpes-like" viral particles similar to those reported by Epstein in his cell lines (EB1, EB2 and EB3). Dr. Manaker is studying EB1 and EB2 cells obtained from Dr. Epstein. Dr. Dalton in collaboration with Dr. Manaker has observed "herpes-like" particles in both cell lines, thus confirming the work of Epstein. Also, he has confirmed the presence of similar particles in one of the human myeloid leukemia cell lines established by Grace. In collaboration with Doctors Sanford and Evans, LB, Dr. Dalton has found both A and C type viral particles in established mouse cell lines in tissue culture.

The C type particles are present when the cells are grown in media containing horse serum, but not in parallel lines grown in chemically defined media containing no horse serum. These observations held for Earle "L" cells and Dr. Sanford's "High" line, both of which were established initially by explanting normal mouse fibroblasts, but eventually underwent "spontaneous" transformation to malignancy *in vitro*. In a survey of mammary tumors supplied by Doctors Andervont, Heston and Deringer, LB, mature type B particles, as well as A type particles, were found in all but those tumors arising in RIII and Deringer C3H_e mice. In these two instances A type particles, only, were found. Dr. Dalton has also identified as type C the virus associated with the mouse sarcoma recently discovered by Dr. Moloney. The particles were indistinguishable morphologically from those of the Moloney and other mouse leukemia viruses.

Dr. David-Ferreira completed and published his thorough electronmicroscopic study of the mouse hepatitis virus (A-59) discovered and previously reported by Dr. Manaker. Dr. Manaker collaborated with Dr. David Ferreira in this investigation. As already mentioned, Dr. David-Ferreira collaborated with Dr. Stewart in determining the morphology of the new canine virus which she discovered.

Dr. Bucciarelli, a guest worker from Perugia, Italy, is collaborating with Dr. Rabotti in the study of brain tumors in dogs induced with the Rous sarcoma virus. Dr. Dietert, who recently joined Dr. Dalton's staff, has begun a radio-autographic study aimed at following the movement of Moloney lymphocytic leukemia virus RNA from its entrance into the cell to its replication as newly formed virus particles.

Dr. Douglas Anderson, in collaboration with Doctors Manaker and Barile, DBS, has completed an extensive electron microscopic study of two strains of mycoplasma. His results have greatly increased our capability of distinguishing organisms of mycoplasma from virus particles in thin section preparations.

Mrs. E. Mitchell continues her studies of ultracentrifuge pellet material derived from plasma obtained by plasmaphoresis from leukemia patients entering the Clinical Center. Of 96 specimens examined with thin-section elec-

tron microscopy 19 were positive for virus-like particles and 6 were questionable. The larger percent (about 20) of definitely positive specimens as compared with the study reported last year (about 12% positive) is associated with the larger samples obtained by plasmaphoresis and the increased amount of plasma contributing to the pellet specimen in each case.

In the Laboratory of Viral Oncology Dr. Moloney has discovered a new mouse tumor virus which produces solid tumors (rhabdomyosarcoma) at the site of inoculation within two weeks after virus administration. The agent came to light in a routine biological test of a highly concentrated preparation of Moloney mouse leukemia virus. Certain of the recipient mice inoculated when newborn developed tumors at the site of inoculation. The solid tumors can be reproduced in serial passages of the virus derived either from extracts of tumor tissue or from the plasma of tumor-bearing mice. As already mentioned, Dr. Dalton has found this agent to be indistinguishable from mouse leukemia virus morphologically. The agent withstands repeated freezing and thawing and has been found fully active after storage for at least 4 months at dry ice temperature (-76°C). Young mice up to 14 days of age (the oldest yet tested) are susceptible to tumor induction by this virus and tumors have been produced in 100 percent of the mice inoculated among 6 different strains and 2 hybrid crosses. The average latent period varied from 9 to 16 days among the different strains and the time to death-with-tumor from 21 to 42 days. A brief note was recently published by Harvey (Nature, No. 4963, 1964) of similar results obtained in studies carried out in England. Solid tumors arising at the site of inoculation of strong doses of Moloney mouse leukemia virus were said to be transmissible with cell-free fractions, but further details were not given. Whether the virus that causes solid tumors is a variant of the Moloney leukemia virus or a different agent that has been picked up and now "contaminates" Moloney virus preparations remains to be determined. The new agent is not neutralized by concentrations of specific anti-serum which completely inactivate the Moloney mouse leukemia virus, but a delay in the latent period of tumors arising after incubation of

mixtures of the virus and anti-Moloney mouse leukemia virus antiserum suggests that the two viruses have some antigenic similarity. In further studies of the mouse leukemia virus which he isolated from sarcoma 37, Dr. Moloney has increased the biological activity by selected serial passage and has demonstrated that, as with the Rous sarcoma virus, the amount of virus recoverable from diseased animals is quantitatively related to the infecting dose of virus. Dr. Moloney has also developed a more rapid bioassay for his mouse leukemia virus, using the weights of spleens 28 days following virus inoculation.

Dr. Gordon Thielen (on sabbatical leave from the University of California) has been studying the avian virus of Twiehaus which was originally isolated from a spontaneously diseased turkey, but which also causes a malignant endotheliosis in chickens. He has found that similar disease is induced in Japanese quail, and quantitative dose-response relationships of this agent have been studied in chicks. Dr. Thielen has collaborated with Dr. Zeigel in electron microscopic studies of the Twiehaus virus, and with Dr. Dalton in a search for viral particles in specimens from leukemic cows. Particles of the C type have been seen in lymph nodes from one leukemic cow. Dr. Thielen has also participated in a collaborative study of animals, particularly dogs, associated with cases of human leukemia. About 10 plasma specimens obtained by him are now being studied by electron microscopy.

Dr. Ruth Merwin has obtained additional evidence that the diffusion chamber method developed by her for the detection of small amounts of tumor virus is a valuable new procedure for bringing to light smaller amounts of such viruses than can be detected by conventional extraction and inoculation procedures. As previously reported, a virus isolated by this method from sarcoma 37 produced both leukemia and "bone lesions" resembling osteopetrosis of chickens. In further studies Dr. Merwin has shown that the Moloney mouse leukemia virus, likewise isolated from sarcoma 37, also induced "bone lesions." It is probable that Dr. Merwin's isolate is the same as Dr. Moloney's. Experiments to determine this are in progress. The induction of bone lesions by

the Moloney virus had not previously been detected. In continuation of her studies on the induction of plasma cell tumors in BALB/c mice by intraperitoneal implantation of plexiglas and other membranous discs, Dr. Merwin has observed hyperplastic plasma cells distributed over the peritoneal surfaces as early as 3 months after implantation of the discs. Grossly diagnosable plasma cell tumors did not appear until 6 months after implantation of the discs.

Dr. Rauscher has carried out quantitative studies on the growth curve of his mouse leukemia virus in BALB/c mice. Virus could not be recovered through the 3rd to 4th day after virus inoculation, but beginning with the 5th day virus was present in both plasma and spleen tissue in relatively large amounts. It increased with time at a regular rate until a peak was reached which was dependent on the dose of virus inoculated. The time to peak was also a function of initiating dose, being 21 days with the 10^{-5} dilution of virus (about 5 ED50). At this lower initiating dose level virus could not be demonstrated in plasma or spleen in about $\frac{1}{3}$ of the animals sacrificed 48 days after inoculation. The results are comparable to those that have been obtained with Rous sarcoma virus and indicate that, as in Rous sarcoma, the neoplasia is viral dependent. Similar studies in rats and in C57BL mice showed the growth curve of virus to be comparable, but the curve was more extended in time in rats. In this host the eclipse phase in which no virus could be detected was 18 to 22 days. The response of C57BL mice was comparable to that of BALB/c during the initial stages, but in C57BL hosts the virus declined progressively after reaching its peak, in contrast to its continued high level through life in the BALB/c strain. A characteristic of the Rauscher virus which distinguishes it from other mouse leukemia viruses is its ability to stimulate erythropoiesis and induce marked splenomegaly within 10 to 14 days following virus inoculation. Lymphocytic leukemia appears later, after 2 or 3 months. The initial manifestations of the biphasic disease do not appear in rats. After passage in rats the recovered virus also fails to induce erythropoiesis and splenomegaly when

inoculated back into mice, although leukemogenic activity is retained. These results suggest the possibility that the original virus material isolated by Dr. Rauscher may have consisted of 2 different agents. This is supported by additional studies of Dr. Rauscher in which the virus passaged in tissue culture lost its ability to induce erythropoiesis and splenomegaly when inoculated back into BALB/c mice. However, all attempts to isolate and propagate the erythropoietic factor separate from the leukemogenic agent have failed thus far. Furthermore, virus recovered from leukemic rats of the 10th serial passage in rats unexpectedly induced marked erythropoiesis in addition to leukemia when inoculated back into BALB/c mice. The question of 2 agents, or modulations involving a single agent, cannot be resolved at this time. Dr. Rauscher has continued his collaboration with scientists of other disciplines in extensive studies on the chemical, immunological, biophysical and electron microscopic characteristics of his virus isolate. He has provided the essential, parallel, quantitative biological and virological components of the joint investigations.

Dr. Zeigel in collaboration with Doctors Rauscher, Fink and Tyndall (Oak Ridge National Laboratory) has carried out further studies on the Rauscher virus. A significant amount of virus is produced by budding from cell membranes and evidence has been found that there is a concurrent phagocytosis of viral particles by the same cells. This suggests a "dynamic equilibrium" with regard to free virus in culture fluid (i.e., virus titer). A systematic electron microscopic study is being made by Dr. Zeigel of various tumors of BALB/c mice. "A" type particles which bud from modified endoplasmic reticulum have been found in all tumors examined thus far. These include an ascites form of Rauscher leukemia cells, a spontaneous solid tumor which arose in mice inoculated with Rauscher virus, two epitheliomas, both primary and transplanted plasma cell tumors, a spontaneous mammary tumor, two myoepitheliomas, a methylcholanthrene induced epithelioma and a spontaneous lymphocytic tumor. In addition to the Rauscher virus lesions, several of the other tumors also con-

tained "C" type particles indistinguishable from those associated with mouse leukemia. Studies on the CELO virus (a chicken virus) have confirmed its structure as an adeno-like virus possessing 252 capsomeres and Dr. Zeigel now regards it as probably identical with the Gal virus (*Gallus* adeno-like virus). These studies were carried out with the collaboration of Dr. Sarma (formerly of NIAID). In collaboration with Dr. Theilen, Dr. Zeigel has studied the Twiehaus virus and found that the particles are not identical with other known strains of avian lymphomatosis, myeloblastosis, and erythroblastosis viruses in electron microscopic morphology. He is continuing his basic studies on normal development of thymic, liver, and pancreatic cells and is collaborating in the Institute program on human leukemia through periodic examinations of plasma specimens of monkeys inoculated, when newborn, with candidate agents derived from human leukemia.

The development of fluorescent antibody techniques applicable to mouse leukemia viruses and to candidate viruses from human leukemia and lymphoma were reported last year by Doctors Fink and Malmgren (LP). Dr. Fink has carried out additional studies on human leukemia using fluorescent antibody prepared in both rabbits and monkeys against the pellet fraction derived by differential centrifugation from plasma of leukemic patients which had been found by Dr. Dalton to contain particles having the ultrastructural characteristics of viruses. As controls, comparable serum reagents were prepared against fractions derived from plasma of normal humans and from plasma of leukemics containing mycoplasma (PPLO) but no virus-like particles. In addition, fluorescent antibody was prepared against several known strains of PPLO, including strains isolated from leukemic patients. Such organisms are frequently present in blood and bone marrow specimens from human leukemia patients. None of the fluorescent antibody reagents prepared against normal plasma fractions or against isolated strains of PPLO have been found to react with leukemic cells from the buffy coat or bone marrow. On the other hand, Dr. Fink continues to obtain positive reactions in a significant number of cases

when buffy coat and bone marrow cells of human leukemic patients are tested with the fluorescent antibody reagent prepared against selected, particle-positive, leukemic plasma fractions. In her studies on a case of Burkitt lymphoma admitted to the Clinical Center, cells from the original tumor biopsy were negative but later became positive for the specific antigen after propagation in tissue culture (by Dr. Stewart). As reported previously, two cell lines of Burkitt lymphoma established by Epstein were found by Dr. Fink to react positively with the human leukemic plasma pellet reagent. Several impression smears of primary biopsy material from Burkitt lymphomas were sent to Dr. Fink by Dr. Epstein. These were also negative. It should be noted also that Dr. Dalton has failed to find virus particles on electron microscopic examination of primary biopsy specimens of Burkitt lymphoma, whereas "herpes-like" virus particles have been observed after the cells are propagated in tissue culture. Three additional cases of erythroleukemia have been studied by Dr. Fink. Bone marrow specimens of two of them were obtained from Dr. Holland of the Roswell Park Memorial Institute and one from Dr. Lee of Maimonides Hospital, Brooklyn. The cells of all three specimens were positive when tested with fluorescent antibody against the Rauscher mouse leukemia virus (which also induces erythropoiesis). This brings to 4 the number of human erythroleukemia cases that have been tested to date and all four have been positive with Rauscher virus reagent. The specimens were also positive with the anti-human-leukemia reagent, but less strongly so. No positive results were obtained with any of the control fluorescent antibody reagents. In collaboration with Dr. Karon (MB) a group of 14 selected Clinical Center patients have had repeated bone marrow examinations. From this longitudinal study it appears that the reaction with the anti-human-leukemia antibody is present when the disease is active and is not present when the patient is in remission. The immunofluorescent technique as applied to cells of the peripheral blood and bone marrow of leukemic humans is believed by Dr. Fink to be detecting an antigen associated with leukemia. The correlation between the presence of "virus-like"

particles in the plasma, and immunofluorescence of leukemic cells from the same patient, supports the hypothesis that the "virus-like" particles revealed by thin-section electron microscopy are associated with the disease. Considerable evidence has been accumulated which tends to rule out the participation of PPLO antigen in the positive fluorescent antibody reactions observed in leukemic cells.

This immunofluorescent technique may prove useful as a screening procedure in addition to electron microscopy for detecting candidate materials for biological tests in monkeys, attempts at tissue culture propagation, etc.; and may provide a clinical diagnostic aid to predict relapse of patients before relapse actually occurs. The emergence of the ability to react by immunofluorescence in lymphoma cells in tissue culture, concurrently with the visualization of virus by electron microscopy, suggests that in *in vitro* systems, beyond the host's defensive environment, virus may be produced in detectable quantity.

The work in the macromolecular Biology Section of the Laboratory of Viral Oncology can be summarized in three areas: Macromolecular interactions; studies of nucleic acids, their base sequence "homology" and their role in protein synthesis in normal tissues and in tumors; and, the nucleic acids of animal tumor viruses including their chemistry and electron microscopy.

In *macromolecular interaction studies*, Dr. Shifrin has continued his research on charge transfer complexes. The interaction of biological molecules by charge transfer complex formation has often been suggested but a definitive study has not yet been carried out on molecules of biological significance. Dr. Shifrin is pursuing a systematic study of a carefully selected model system pertaining to enzyme-coenzyme and enzyme-substrate interaction, specifically, the complex formation between nicotinamide adenine dinucleotide or thionicotinamide adenine dinucleotide and certain electron donor molecules. He has found a linear relationship between the ionization potential of the electron donor and the energy of the intramolecular charge transfer transition. This linear relationship is an empirical demonstration of quantum mechanical calculations, and it can be used

to predict the position of any charge transfer band, knowing the ionization potential of a donor molecule. What is more important biologically, one can predict from the position of a charge transfer band the ionization potential and, therefore, the exact nature of a donor molecule. Dr. Shifrin has extended his study of charge transfer bands to molecular complexes in the absence of light. His current studies, which include collaborative research using electron spin resonance spectroscopy, now pertain more broadly to the interaction between amino acid side chains and the nucleotide bases. For the objectives of pursuing studies on the effect of changing the acceptor molecule from nicotinamide to thionicotinamide, and on how this change affects the charge transfer transition, Dr. Shifrin developed new organic chemical synthetic methods for the preparation of certain substituted thiocarbamoylpyridinium chloride derivatives. His studies provide further insight into the nature of the enzyme-coenzyme complexes and the enzyme substrate complexes. In addition, they give information on the direct interaction between the bases in polynucleotides and the various amino acids in polypeptides.

Dr. Mora in collaboration with Dr. Philip Person (VA Hospital, Brooklyn, New York) found that the oxidized form of cytochrome c changes into the reduced form upon adding a polycation to the pure cytochrome c. If an excess of polyanion is then added, the reduction is reversed and the cytochrome c can be recovered again in the oxidized form. These reductions and oxidations can be repeated in cyclic fashion and they occur at a characteristic rate depending on the polyions employed. It appears that beside the polycation nature of the substances which act as reducing agents, these also must have hydrophylic characteristics. Some of the substituted cationic polyglucosidic derivatives were found to be most effective reducing agents, although many other physiologically occurring cationic biopolymers (histones, etc.), are also quite effective. It should be emphasized that the effects of the polyions are exactly opposite to that which one would expect from direct electron transfer. In studies on the mechanism of the reduction Drs. Mora and Person found that by heating, or by treatment

with alcohol, the reduction effect of the polycations could be reversed. The oxidized form is apparently the more stable and more open tertiary structure.

Dr. Mora also discovered a similar reduction of cytochrome c by certain sugars, for example by glucoseamine, galactoseamine or ribose. It is of interest that certain other, more reducing, sugars and derivatives such as glucose do not have this reducing effect on cytochrome c. Both the polycation effects and the carbohydrate effects apparently represent a mechanism for controlling reducibility or oxidizability of the cytochrome c through changing the tertiary structure of cytochrome c, thus operating as an "allosteric control." These findings allow a deeper understanding of oxidative phosphorylation in the mitochondria. Since in all types of cells, animal and plant, the terminal electron transport and oxidative phosphorylation are mediated by the cytochrome cycle, and the energy of ATP is utilized by this process in the mitochondria, it is of importance to determine the conditions which control these processes.

In research on *nucleic acids in protein synthesis*, Dr. Luborsky, in collaboration with Drs. Sidney Petska and Marshall Nirenberg (NHI), studied the sedimentation properties of ribosomes obtained from a streptomycin sensitive strain of *E. coli*. It had been observed previously that addition of streptomycin to the Nirenberg cell-free amino acid incorporating system induces errors in the amino acid incorporation as directed by a messenger RNA. Drs. Luborsky and Pestka obtained results which indicate that the "ambiguity" errors probably arise from the fact that the template activity of the messenger RNA in the protein synthesis depends on the meditation of an active 70s ribosomal species to maintain the activated aminoacyl-sRNA in a proper position for the formation of the peptide bond.

Before a DNA duplication or transcription process may begin, at least a partial opening of the double stranded DNA structure is obligatory. The influence of polyanion and various conditions of pH and temperature on strand separation was studied by Dr. Mora on bacteriophage T1 DNA. He found that the DNA helix \rightleftharpoons coil transition occurs at lower pH, at lower temperatures, but polyanions do not affect

either the pH transition or the temperature transition. By inference, it can be deduced that the DNA molecules do not influence each other in their transition, even when they occur at a very high localized concentration, such as in the nuclei of cells. Thus, DNA-DNA electrostatic interactions do not participate in control processes of DNA duplication and DNA transcription.

Dr. Carl Smith is engaged in long-range studies on mammalian messenger RNA, using nucleic acid homology techniques. These techniques involve the separation of the two nucleic acid strands from the double helix (by the same heating technique which Dr. Mora studied on the bacteriophage T1 DNA) into single stranded structures, and hybridizing these strands with the complementary mRNA which selectively adsorbs onto the DNA. The adsorbed mRNA can then be fractionated away from other RNA species such as the ribosomal and sRNA. Demonstration and measurement of the base sequence complementarity in various mRNA molecules is thus possible. DNA-DNA hybrids can also be similarly worked with. The normal rat liver and the Morris hepatoma 5123, which is a minimal deviation hepatoma were selected by Dr. Smith for his studies. He has developed efficient fractionation procedures for both the liver and the hepatoma which permit the separation of nuclei (where the DNA controlled mRNA synthesis occurs) from the cytoplasmic fraction (where most of the protein synthesis occurs). To obtain DNA, he used the extraction procedure of Marmur, which he modified for his purpose. With these modifications DNA was obtained in the 12-14 million molecular weight range. It was possible to trap the DNA in an agar gel network used to support the single stranded DNA during the homology experiments. To obtain quantitative data the specific radioactivity of the nucleic acids must be very high. In studying the conditions of injection of p^{32} orthophosphate, which is then incorporated into both DNA and RNA, Dr. Smith studied conditions for obtaining liver cytoplasmic RNA and also hepatoma cytoplasmic RNA with high radioactivity. The RNA preparations were obtained by cold phenol extraction, both from the nucleic fraction and from the cytoplasmic frac-

tion. These were then hybridized with the unlabelled DNA, in separate experiments from liver and from hepatoma. Quantitative conclusions regarding RNA to DNA homology must await further experimental development aimed at increasing the radioactivity of the mRNA. However, the DNA-DNA homology studies showed that the liver and hepatoma cells are alike in the nucleic acid sequence in their DNA.

In the *studies on tumor-viruses*, Dr. Luborsky, in collaboration with Dr. Bader (FS) has devised methods for the production of high titer Rous sarcoma virus in moderately large amounts in tissue culture. Dr. Luborsky also developed methods for concentrating and purifying this virus in a stable, infective, form in serum-free medium. This was necessary before he could proceed with the investigation of the biochemical properties of the virus and its mode of action in producing neoplastic transformation of cells. His success involved the use of a "cushion" of potassium tartarate solution to collect the virus during sedimentation in an ultracentrifuge and further fractionation in a potassium tartarate density gradient. With these methods 46% of the input infectivity was recovered with more than a 100-fold increase in concentration. The infectivity studies were carried out by Dr. Bader. One further purification step, involving the use of sephadex G 200, was necessary for separating the virus from low molecular weight contaminants. By these combined methods, Drs. Luborsky and Bader also succeeded in preparing highly purified tritium labelled Rous, and Rous-associated, viruses. They also observed during these studies that most of the radioactivity could be recovered if certain plastics, tubes, rather than glass tubes, were used for centrifuging the solutions. The virus was found to adsorb readily to glass, but not to the plastics. Sedimentation properties of the Rous virus yielded estimates of about 600s for a sedimentation coefficient in ultracentrifuge studies. This value is in line with a 80 m μ diameter, 1.16 g/ml density, particle with a total weight of 1.9×10^8 , determined by independent measurements.

Dr. O'Connor has continued his studies in collaboration with other members of the Laboratory of Viral Oncology (Drs. Rauscher, Mo-

loney, Fink, and Manaker). Certain physical-chemical, biochemical and immunological properties of animal leukemia viruses and their subviral fractions were investigated, especially from the point of view of developing leads that will be useful in the search for possible viruses that might be involved in some human leukemia. He employed density gradient studies extensively both to characterize these viruses and to separate the subviral components obtained after various partial degradative treatments. In density gradient studies, the Rauscher virus, Moloney virus, and Friend virus appear to have the same buoyant density 1.16 g/ml, a value also obtained by Dr. Luborsky for the Rous sarcoma virus. In addition, Dr. O'Connor has studied influenza virus (PR8 strain), the Newcastle disease virus, and the mouse mammary tumor agent by centrifuging mainly in potassium citrate gradients. Avian myeloblastosis virus was found to disintegrate in potassium citrate, but it was successfully banded in a sucrose gradient. The density of this virus was determined to be 1.15 g/ml. A large amount of Dr. O'Connor's effort has been directed toward the development of a controlled degradation procedure for the animal leukemia viruses, especially the Rauscher virus. Detergents, or ether were used for degrading the virus, and the subviral components were separated by density gradient centrifugation. The lipid rich components have a low density, while the lipid poor components, which contain more of the nucleoproteins, are heavier. These various components were examined by Dr. de Thé with the electron microscope and are now under study by Dr. Fink using fluorescent antibody methods. Similar studies are being applied also to the Moloney virus and to the Newcastle disease virus. These studies on subviral components are directed toward the identification of possible human leukemia viruses, as well as toward a determination of the relationships of the various animal leukemia viruses. Close liaison is maintained between this project and the research of clinicians and biologists working on animal leukemia viruses. Density gradient fractionation techniques already developed are now being employed on both plasma and cell extracts of selected human leukemia cases, with the objective of obtaining

higher quantities of the "virus-like" particles present therein.

The properties of the nucleic acids in the various murine leukemia viruses are of importance for studies on their mode of action. For example, it is necessary to know whether the nucleic acid (RNA) is single stranded or double stranded, and whether or not it behaves as a messenger RNA. Characterization of the Rauscher virus nucleic acid was undertaken by Dr. Peter Mora. He found that the Rauscher virus contains only RNA in detectable amount, and that the RNA consists of only the normal nucleotides of U, A, G, and C. It is very sensitive to RNase digestion which causes it to split easily during the isolation process into lower molecular weight products. This indicates a single stranded RNA rather than a double stranded form. Single strandedness was further indicated by experiments on helix \rightleftharpoons coil transition studies by heating and cooling, in techniques similar to that carried out by Dr. Mora on the DNA of T1 bacteriophage. Since these findings indicate that the Rauscher virus RNA is single stranded, there is no need to assume a double stranded RNA structure as a *necessary property* of the RNA of cancer viruses, as has been recently suggested by others.

Dr. Guy de Thé has carried out electron microscopic studies on certain of the structural properties of viruses and on cell-virus relationships. He has found that ATPase and inosine triphosphatase are present in the virus envelopes of both the Rauscher and Moloney viruses. This is similar to the findings with myeloblastosis virus. However, these activities were not detected in the membranes of channels within the infected cells. The mammary tumor agent was also examined by a similar technique. Low enzymatic activities were detected in this virus and also at the apposed cell membrane of acini, where the virus is formed by budding. The enzymes were also found associated with the Rous sarcoma virus, but only when they were produced *in vivo*; virus produced in tissue culture did not contain ATPase. In collaboration with Dr. Notkins (NIDR), Dr. de Thé has studied the structure of the lactic dehydrogenase virus and found that it has an elongated elliptical shape, 40 by 75 m μ in diameter. They also found the site of

replication of this virus to be in the cytoplasmic vacuoles and at the cellular membranes of peritoneal macrophages. In collaboration with Drs. Luborsky and O'Connor, respectively, Dr. de Thé is also studying the morphology of the Rous sarcoma and Rauscher leukemia viruses and their subviral components.

CLINICAL INVESTIGATIONS

The accompanying reports from the Clinical Branch Chiefs detail the research accomplishments of each Branch. In addition, attention should be called to the following specific items: (1) reverse isolation, (2) intensive care, (3) recruitment of patients for radiation therapy, (4) the creation of the Immunology Branch, and (5) the development of an experimental surgery for the Surgery Branch.

While only four patients have been studied by reverse isolation in an apparatus known as the "Life Island", a term which has been applied to this study, the clinical results have been striking. It has been possible to maintain adults in the chamber for periods of weeks. In fact, a patient has been maintained in the "Life Island" for eight weeks. It has been possible to sterilize effectively the skin and gastrointestinal tract of these subjects. More important, the physicians caring for these patients have been able to use doses of chemotherapeutic agents considerably in excess of what they would consider reasonable in other circumstances. This is because of the astonishing decrease in toxicity to the gastrointestinal tract or at least toxicity as manifest by ulcerations in the entire gastrointestinal tract. The prevention of infection by fungi and bacteria has also permitted the maintenance of patients in the reverse isolation apparatus without the attendant danger of lethal infection. It is much too early to predict the outcome of these studies. However, it is not too early to state that the changes in toxicity observed are remarkable. It is hoped that this procedure will lead to better understanding of the role of infection in toxicity and to ways of giving patients additional quantities of drugs. It should be mentioned that these studies are very costly in terms of our personnel resources, particularly nursing.

This past year has also seen the completion of an intensive care unit for patients in the Surgery Branch. This will now permit all post-operative patients to be cared for in a special facility designed for this purpose. It will also permit the surgeons to care for other patients in this facility when the need is indicated. This facility has its own nursing staff.

The past year has witnessed the ability of the Radiation Branch to gain referrals of patients suitable for radiation therapy studies. In fact, it has become quite apparent that we can gain enough referrals of patients to support a clinical radiation therapy program. At the present time the principal need is to provide for additional beds for this Branch.

This past year has also seen the creation of the Immunology Branch to meet the needs of the National Cancer Institute to further its research goals in the immunological aspects of cancer.

The last items deal with the assignment of space in Building 14D to the National Cancer Institute for the support of a large animal surgery which will permit the development by the Surgery Branch of a large animal surgery program.

These constitute specific items to which the Clinical Director desires to call attention, the reports of the Branch Chiefs should be consulted for specific details and accomplishments of each branch.

Amino Acid Metabolism

Studies of amino acid metabolism have continued. These studies are designed to elucidate the physiologic and biochemical phenomena responsible for the entry of amino acids into mammalian cells and to clarify the relationships between rates of amino acid transport and subsequent intracellular utilization and to define the significance of defects in amino acid transport in man. Initially this work was conducted *in vitro* using the rat kidney cortex slice as the tissue model. However, during the past year several other tissues have been studied and one of the surprising findings has been the distinct differences noted in amino acid transport mechanisms between different tissues both in man and the experimental animal. For

instance, alpha aminoisobutyric acid, a model amino acid which is actively transported by the kidney, is not concentrated by intestinal mucosa despite the anatomic similarities in these two tissues. Secondly, in the cystinuric kidney no defect in cystine transport has been demonstrated whereas a marked defect in cystine uptake was shown in the intestine. Furthermore, human leukocytes from cystinuric patients showed no defect in transport of lysine or cystine in contrast to the results in kidney and intestine. These and other findings suggest that there are important biochemical differences in cell membranes structure in different tissues, and that indeed, mechanisms of genetic control are also different in different tissues of the body.

The role of the sodium ion in amino acid transport has been studied for the past two years. It has been shown in many tissues that removal of the sodium ion markedly interferes with mediated transport of a variety of amino acids. This sodium dependence has been studied in the intestine, kidney, leukocytes and rat bone. These studies indicate that the sodium ion may have more than one role in amino acid transport. It has been well shown that the transport of sodium across the cell membranes is an important regulator of cell respiration and hence, the coupling of transport mechanisms to energy may well be regulated via sodium transport. In addition, however, the sodium ion has been shown to be necessary for transport systems in the absence of metabolic energy indicating a direct role in the passage of amino acids across the membrane region. Our studies have shown that the sodium ion stimulates influx of amino acid into the cell rather than affecting the efflux process and that the increase in movement of amino acid in the presence of the sodium ion is associated with an increase in net water movement across the intestinal wall. The phenomena of sodium dependence has also been used to study transport mechanisms for the neutral amino and imino acids in bone in the following way. Bone cells, or slices, incubated in the presence and absence of the sodium ion demonstrated that proline and hydroxyproline showed a complete dependence on the sodium ion for entry whereas glycine and alanine were only partially sen-

sitive to this ion. Furthermore, when sodium ion was removed from the incubation medium, proline and hydroxyproline no longer inhibited glycine entry nor was the entry of proline affected by glycine or alanine. These studies show clearly that the amino and imino acids have different transport mechanisms and indicate that the sodium ion may indeed enter into linked flux relationships with amino acids and that these sodium-amino acid complexes in the cell membrane differ for different amino acids.

We have continued to explore the nature of the transport defects in cystinuria, and have been led to the conclusion that cystinuria is indeed two distinct diseases. This conclusion is based on the following evidence: first, two patients with documented homozygous cystinuria showed normal transport of cystine and lysine by gut mucosa in contrast to the severe defect shown in twelve other cystinuric patients. These *in vitro* findings were corroborated by oral loading studies with cystine which indicated that those cystinuric patients who could transport cystine *in vitro* were also capable of absorbing cystine from the gut while those subjects who failed to transport *in vitro* were unable to absorb cystine to any significant degree. Additional evidence for genetic difference was made available through study of families of the cystinuric patients. These studies showed that the cystinuric subjects who were capable of transporting the dibasic amino acids and cystine by the intestine could be identified in the families by hyperexcretion of lysine and cystine in the urine while no urinary abnormalities could be demonstrated in the families of those patients with the severe gut lesion. This leads to the conclusion that cystinuria must result from two separate autosomal defects with similar or identical urinary amino acid findings but with different manifestations regarding the intestinal tract and mode of genetic transmission. We have also investigated further the puzzling observation that cystine transport in the human cystinuric kidney *in vitro* was not defective. This led to the fundamental question of why these people excreted so much cystine. Renal arterio-venous differences were performed in two cystinuric patients and essentially no arterio-venous difference for cystine or cysteine were demonstrated in either case.

These results are not compatible with a simple failure of tubular reabsorption of cystine by the kidney and suggest that the cystine excreted in large quantities in cystinuria may well be formed in the kidney by defective intrarenal metabolic processes.

Calcium Metabolism

During the past year the analysis of calcium kinetics in normal subjects and patients with metabolic bone disease has continued. In this work, calcium balance studies have been combined with Ca^{47} disappearance from the blood and appearance in urine and feces. In addition, Ca^{47} has been monitored externally over the thigh and ankle. The data have been analyzed using a digital computer multicompartmental approach. The results in ten normal young men provide the first homogenous group of normal subjects studied this extensively. They indicate that in the period from 1 minute to 20 days after intravenous injection, Ca^{47} exchanges with four body pools which do not have clear anatomic definitions. These studies provide quantitative estimations of the rate of transfer of calcium between these compartments as well as the rate of movement of stable calcium from the gut into the body, from the blood into the intestine, from the blood into the urine and from blood and tissue compartments into non-exchanging bone. The mathematical analysis has been programmed to yield numerical values for the uncertainties in the various parameters so that future studies of metabolic bone disease will have a suitable frame of reference. The results obtained with this compartmental analysis have been compared with those obtained using a simple one compartment model. This comparison has demonstrated that marked discrepancies result if only a single compartmental model is used. Three patients with osteopetrosis or marble bone disease have been studied. These patients show a markedly enlarged miscible calcium pool. However, even more striking has been the curves of appearance of calcium over specific bone sites in the osteoprotic patients. These studies reveal a marked slowing of removal of calcium from the specific bone sites and analysis to date suggests that this slowing may be due to a specific decrease

in the rate constant for movement of calcium from the most slowly exchanging calcium compartment. These results would be compatible with the histologic findings suggesting the retention of calcified cartilage matrix as an important feature in the pathogenesis of this disease. Additional studies are in progress to determine whether age, sex or diet influence the measured parameters.

Nucleic Acid and Pyrimidine Metabolism

In continuation of the studies concerning the elevated urinary pseudouridine uric acid ratio found in chronic lymphatic leukemia patients, investigations were performed on the ribonucleic acid formed by normal small lymphocytes and small lymphocytes from patients with chronic lymphocytic leukemia. It was found that under the incubation conditions employed, the rapidly labelled ribonucleic acid had the anticipated high sedimentation coefficients of 35 to 45 S and were in large percentage hybridizable to human cell DNA. There was very slow conversion of only a small portion of this RNA to ribosomal nucleic acid unlike the events seen in dividing cells, such as KB cells. Very small amounts of S-RNA were synthesized. Thus, it is difficult to account for the excess pseudouridine production on the basis of RNA production by the small lymphocytes.

Further studies of lymphocyte metabolism indicated that these cells are producing significant amounts of major classes of histones even when they do not produce DNA. Investigations of histone fractionation by preparative acrylamide electrophoresis are continuing.

Negligible amounts of polyuridylic and polyadenylic have been found to occur naturally in *E. coli* when searched for by hybridization techniques

Studies are continuing on the phenomenon observed in KB cells in which, under proper conditions azaguanine is incorporated solely into 4S transfer RNA.

Nucleic acid hybridization studies of biologic phenomenon are continuing in several directions. Applications of the methods to classification of mycoplasma have indicated that the method has great sensitivity in detecting strain differences among organisms and gives an in-

dependent method of classifications of these organisms that in most conditions agrees with the serologic classification.

Efforts are continuing to detect viral DNA in viral induced tumors. Studies of RNA production in adenovirus infected cells indicate that viral messenger RNA is produced in the cells that are continuing to produce their own host-complementary RNA. In addition a component of RNA similar but chromatographically distinct from transfer RNA appears in adenovirus infected KB cells.

Studies of hemoglobin biosynthesis by normal reticulocytes have indicated a lag of incorporation of radioactivity into the alpha as compared to the beta chain or finished hemoglobin suggesting accumulation of precursor pool of alpha chain material.

In thalassemia the situation is reversed in long term incubations and the alpha chain of finished hemoglobin has a higher specific activity than the beta or gamma chains consistent with the hypothesis of alpha chain overproduction in these patients.

Studies of plasma protein synthesis in hepatectomized dogs and monkeys, have indicated small but quite significant amounts of synthesis of alpha and beta globulins in these animals.

The Metabolism of Plasma Proteins

The technique for the direct measurement for the absolute synthetic rate of liver-made proteins using carbon¹⁴ labelled bicarbonate was compared to the indirect method of measuring protein synthetic rate using I¹³¹ labelled proteins. The latter method is valid under steady state conditions. The rate of albumin synthesis was estimated by both methods in six subjects without serum protein abnormalities and five patients with protein losing gastroenteropathy. There was a very close agreement between the estimates of synthetic rates by both methods. It is therefore felt that the technique for the direct measurement of protein synthetic rate developed will be of considerable value in the study of the factors controlling protein synthesis in man.

The metabolism of albumin, gammaglobulin (IgG), B₂A globulin (IgA) and gamma macro-

globulin (IgM) was studied in a group of 10 patients with hypogammaglobulinemia due to defective synthesis. This group of patients included subjects with congenital defects in gamma globulin synthesis, idiopathic acquired hypogammaglobulinemia and hypogammaglobulinemia secondary to thymomas or chronic lymphocytic leukemia. In each of the patients the primary disorder of protein metabolism was a defect in the synthesis of the three immunoglobulins. Four of the patients had an associated gastrointestinal disorder with secondary gastrointestinal protein loss and a short survival of each of the proteins. Each of the remaining patients had a normal survival of albumin, macroglobulin (IgM) and B₂A globulin (IgA) but had a markedly prolonged survival of gammaglobulin (IgG). This finding is in accord with the hypothesis that there is an indirect correlation between the survival half time of IgG and the serum concentration of this protein. This finding appears to be best explained by the presence normally of a saturable system protecting IgG molecules from catabolism. As the concentration of IgG molecules decreases a larger fraction of the total number of IgG molecules are protected by this saturable system and the survival of IgG is prolonged.

The metabolism of albumin and the three major classes of immunoglobulins was studied in a group of 18 patients with myotonia dystrophica. In these patients the serum concentration and rates of synthesis and catabolism were normal for albumin, IgA, and IgM. However, the majority of the patients had a significantly reduced concentration of IgG globulin. It was found that the catabolism of normal IgG was accelerated in these patients with half times averaging 12 days compared to over 24 days in 17 normal control subjects. IgG obtained from patients with myotonia dystrophica had a similar short survival in these patients but survived normally in control subjects. The rate of synthesis of this protein, however, was normal. The reduced serum protein was thus entirely accounted for by an increased rate of breakdown of IgG molecules. No such hypercatabolism of IgG was noted in approximately 20 other patients with neuromuscular diseases. Thus patients with myotonia dystrophica ap-

pear to have a unique immunoglobulin abnormality an isolated hypercatabolism of IgG.

The metabolism of the immunoglobulins was also studied in patients with ataxia telangiectasia. This disease is characterized by progressive cerebellar ataxia, sinopulmonary infection, oculocutaneous telangiectasia, skin anergy, thymic abnormalities, and in a significant number (8 of 40) of cases associated reticuloendothelial malignancies. The immunoglobulin levels in seven such patients were studied. The major abnormality was an almost complete absence of IgA globulin in six of the subjects and a reduced level of this protein in the remaining patient. Each of the patients had a normal survival of IgG, IgM and albumin. Each of the five patients studied had an extreme defect in the synthesis of IgA globulin. Four of these patients had in addition an exceedingly short survival of IgA globulin indicating that combined defects in both IgA synthesis and catabolism were present. It was demonstrated that the patients with ataxia telangiectasia with an absence of IgA in the serum had significant quantities of IgA in their saliva. It was shown by immunofluorescent studies that there were significant quantities of this protein in the salivary acinar cells. This finding is in accord with the observation of Tomasi and co-workers that there are two related IgA type proteins, one formed in the plasma cells and the other formed in excretory tissues such as the salivary glands. It is presumed that in these patients there is a defect in the plasma cell but not of the salivary IgA globulin synthesis. The association of disorders of the thymus, the disorders of immunoglobulin metabolism and the frequent occurrence of malignant tumors in these patients presents an interesting area for speculation on the role normally played by the immunological system in the prevention of neoplastic disease.

Studies previously initiated in patients with excessive gastrointestinal loss of protein were continued. Patients with gastrointestinal protein loss were shown to have a markedly reduced level of albumin, IgG and IgA, a moderate reduction of transferrin, IgM ceruloplasmin and normal levels of fibrinogen. Using iodine labelled proteins it was shown that there was a marked increase in the fractional catabolic

rate for albumin, IgG, IgA, IgM and ceruloplasmin in patients with gastrointestinal protein loss. In general, the increase in the fractional catabolic rate over normal was quite comparable for the different proteins for a given patient, suggesting that there was bulk loss of plasma or of material with a comparable protein composition into the intestinal tract in these subjects. The synthetic rate for IgG, IgM was normal or slightly increased in each of the patients studied with the exception of one patient with regional enteritis who had high synthetic rate for IgC. This suggests that a reduced serum concentration of the immunoglobulins is not a potent stimulus for their synthesis and that other factors such as exposure to antigens are the major stimuli to immunoglobulin production.

A combined technique for the study of patients with protein losing enteropathy was introduced using the simultaneous administration of I^{125} labelled albumin and chromium⁵¹ labelled albumin. Using these combined studies the plasma volume, protein pool sizes, the rate of protein catabolism, the fraction of the intravascular pool lost into the intestinal tract daily, and in the steady state the synthetic rate can be determined. In normal subjects it was found that approximately 2 to 10% of the albumin catabolism could be explained by gastrointestinal protein loss. In patients with increased gastrointestinal protein loss, 60% of the circulating albumin pool could be lost into the gastrointestinal tract each day. The major disadvantage in the technique is that chromium⁵¹ is gradually eluted from the serum protein. Because of the short apparent survival of chromium⁵¹ labelled albumin, copper⁶⁷ labelled ceruloplasmin has been developed as a technique for quantitating gastrointestinal protein loss. This material was evaluated in studies in dogs, normal man and patients with gastrointestinal protein loss. This material appears to fulfill better the major requirements for an ideal label for the quantitation of gastrointestinal protein loss than any previously proposed material. The radioactive copper is incorporated into ceruloplasmin and becomes an integral part of the molecule throughout its life span without altering its metabolism or distribution. There is relatively little absorption of the ra-

diolabel from the intestinal tract and there is a relatively low level of excretion of the label into the intestinal tract unbound to the serum protein. The survival of copper labelled ceruloplasmin and I^{131} ceruloplasmin were comparable in all groups studied. In normal dogs and man less than 20% of the overall metabolism of this protein could be explained by loss into the gastrointestinal tract. Thus loss of ceruloplasmin into the intestinal tract plays only a minor role in the metabolism of this protein in normal subjects. In contrast in patients with gastrointestinal loss such loss of ceruloplasmin is a major factor in the metabolism of the protein.

During the past year gastrointestinal protein loss has been demonstrated in patients with constrictive pericarditis, regional enteritis, Whipples disease, sprue, celiac disease, carcinoid tumors, generalized amyloidosis associated with neoplastic disease, agammaglobulinemia, and patients with gastrointestinal allergy as well as in new subjects with the syndrome of intestinal lymphangiectasia. Further studies of patients with intestinal lymphangiectasia have revealed multiple immunological defects in these patients. In addition to the short survival of the immunoglobulins these patients have marked lymphocytopenia, they have absence of tonsillar tissue and have skin anergy as well as the ability to retain skin grafts for long periods.

Previous studies in man suggested that catabolism and to a lesser extent urinary loss contributed to the metabolic fate of Bence-Jones proteins. In these studies, patients with normal renal function had a much higher catabolic rate for Bence-Jones proteins than those patients with an elevated blood urea nitrogen. In the present study the role of the kidney in the catabolism of Bence-Jones proteins, isolated L-chains of IgG proteins and intact immunoglobulin was studied in mice. Bence-Jones proteins of the L-chains type from both human and mouse origin were catabolized at a much slower rate in nephrectomized than in ureter-severed or control mice. Similarly, the catabolism of the L-chain fraction IgG molecules was markedly reduced by nephrectomy. There was no difference in the rate of catabolism of whole IgG, IgM or IgA molecules between the ne-

phrectomized and ureter-severed mice. Thus the indigenous metabolism of Bence-Jones proteins and of the L-chains moiety of IgG occurs primarily in the kidney. The kidney, however, does not appear to be a major site of catabolism for the intact immunoglobulins.

Porphyrin Metabolism

A new method has been perfected for the chemical determination of aminoketones in very small quantities to (a) study urinary excretion and (b) measure tissue enzyme activity of ALA synthetase, aminoacetone synthetase, and threonine dehydrogenase. A quantitative method for the measurement of delta-aminolevulinic acid synthetase (ALA synthetase) in homogenates has been developed and applied to studies of porphyria and the factors controlling heme synthesis. Using these methods it has been shown that ALA synthetase in mammalian liver is 1) the rate controlling enzyme in heme biosynthesis, 2) an inducible mitochondrial enzyme and 3) an enzyme whose induction is inhibited by glucose.

In a patient with acute intermittent porphyria, it has been shown that the increased excretion of porphyrin precursors is a result of a markedly increased hepatic level of ALA synthetase. This is the first example of an "overproduction disease" which has been localized to a specific enzyme. It is also the first example of the so-called "glucose effect" in man, i.e., the ability of glucose to inhibit the induction of an enzyme.

A new pathologic entity has been discovered in a patient who expired from acute intermittent porphyria. This consists of a specific destruction of the supraoptic and paraventricular nuclei. This is related to the syndrome of "inappropriate secretion of antidiuretic hormone" in acute porphyria which was first reported by this laboratory several years ago.

Studies of the factors involved in the control of heme synthesis and the relationships of this control to the level of heme protein enzymes have been conducted in mammalian liver. Tryptophane pyrrolase (TPO) is a heme containing enzyme which has served as a model system. Injection of allylisopropylacetamide (AIA) leads to induction of ALA synthetase

which has a half life of 67-74 min. as determined by inhibition of synthesis by puromycin. The increased production of heme resulting from induction of ALA synthetase leads to increased saturation of the apoenzyme of TPO by heme followed by an increase in the amount of total enzyme protein. Preliminary data suggest that the increased TPO protein level results from a longer half life of the protein which may result from stabilization of the protein by heme. The induction of ALA synthetase and the rise in TPO level which follow administration of AIA can be prevented by actinomycin indicating enzyme induction is the mechanism of ALA synthetase increase.

Administration of tryptophane causes an increase of the heme saturation (heme binding) of the TPO apoenzyme followed by induction of ALA synthetase. Only the induction of ALA synthetase is prevented by actinomycin. These data suggest that the degree of repression of hepatic ALA synthetase is related to the level of "free" or "available" heme in liver cells. The coordinated control of both heme and apoenzyme levels can thus be maintained following changes in the protein moiety or in the heme synthetic pathway. Increased binding of heme by the apoenzyme diminished the repression of ALA synthetase, allowing for more heme production. Primary induction of ALA synthetase increases heme production which increases the amount of heme bound to the apoenzyme and prolongs its half life, thus raising its level. Preliminary data from other laboratories suggest that the tryptophane pyrrolase system may be a valid model for the study of the coordination of heme and globin synthesis in the marrow.

Studies in adrenalectomized animals have demonstrated that adrenal steroids exert a permissive effect on the induction of ALA synthetase in that injection of adrenal steroids causes no induction, but induction can not occur to a significant extent in their absence.

The presence of a large variety of tumors in animals causes a decrease of hepatic ALA dehydrase with progressive tumor growth. Polyoma induced tumors appear to be an exception to this phenomenon in that decreased hepatic ALA dehydrase is seen only occasionally with these tumors, regardless of tumor

size or hematocrit. Repeated transplantation and the presence or absence of the polyoma virus do not affect the inability of polyoma induced tumors to lower the enzyme. Further studies are in progress to determine which factors are related to the ability of a tumor to lower hepatic ALA dehydrase.

Erythropoiesis

Studies of the control of porphyrin biosynthesis continue. The initial studies of ineffective erythropoiesis in man have been completed. Following administration of isotopically labeled glycine in man, there are two peaks of the incorporation of isotope in the fecal bile pigments. The first peak reaches a maximum in a few days and the second a maximum in approximately 120 days. The second peak represents bile pigment produced from the catabolism of red cells at the end of the red cell life span. The origin of the first peak has not been clear. In the previously recorded studies in patients it was evident that when erythropoiesis was absent by clinical and biochemical tests, that the amount of labeled glycine diverted to the early peak was markedly reduced to the order of less than 10% of that expected in the normal state. In order to study the early peak, bile fistula dogs were prepared and labeled glycine and labeled delta aminolevulinic acid administered. Previous data by Israels indicated that the early peak could be divided into two components. In order to study the early peak in the dog, both glycine-C¹⁴ and ALA-C¹⁴ were administered. With glycine-C¹⁴, but not with ALA-C¹⁴, in the normal dog there was evidence for a second component to the early peak. This component is markedly increased when erythropoiesis is increased as a result of bleeding and is markedly decreased when erythropoiesis is decreased by transfusion to polycythemic levels. Analysis of the data from the normal polycythemic and the phlebotomized dog indicates that in the normal approximately two-thirds of the isotope appearing in the early peak is associated with erythropoiesis; one-third is associated with another source. Studies by Schwartz at the University of Minnesota indicate that this source is primarily the liver and is due to the turnover of

heme containing enzymes principally catalase in the liver. ALA-C¹⁴ was shown to be a very good precursor of labelled bilirubin and is now the method of choice for the preparation of carbon¹⁴ labelled bilirubin. A conversion of approximately 20% of the isotope to bilirubin has been achieved, compared to a yield of approximately one-tenth percent when glycine-2-C¹⁴ is utilized.

Bilirubin Turnover

A method has been developed for the measurement of bile pigment production by the use of the measurement of bilirubin turnover. Bilirubin-C¹⁴ has been prepared from bilirubin in the dog, has been crystallized, sterilized and brought into a physiological complex with albumin and administered to patients. Samples of plasma were taken and the bilirubin separated into free and conjugated forms by the method of Weber and Schalm. The specific activity of the free bilirubin was then determined. The rate of change of specific activity of the plasma free bilirubin could be described in terms of two decreasing exponential components. Those data could be fitted to a simple three compartment model and the turnover of bile pigment calculated. In patients with normal erythrokinetics this ranged from 226 to 277 mgm/day. In patients with other evidence of increased bile pigment production by virtue of a decreased red cell life span, bile pigment production was increased to 600 mgm/day. These are the first measurements of total bile pigment production in man with a normal serum bilirubin, other than by a surgical drainage through a T-tube.

An *in vitro* system for the measurement of hepatic glucuronyl transferase using bilirubin-C¹⁴ as a substitute has been developed. In the rodent a significant difference between male and female levels of enzyme activity has been noted. A small number of measurements have been made in man.

In order to study the relationship between metabolic rate and erythropoiesis an indirect calorimeter has been constructed for the dog, in collaboration with the Metabolic Diseases Branch of the National Institute of Arthritis and Metabolic Diseases. This chamber provides for the measurement of carbon dioxide produc-

tion and oxygen consumption. The chamber is now partially automated. It is anticipated that when fully automated continuous oxygen consumption and carbon dioxide production measurements can be obtained. It has been shown that it is possible to extrapolate the data from a 4 hour run to a 24 hour determination, thus shortening considerably the period required for the measurement of metabolic rate. In the normal dog the metabolic rate average ranges from 40-50 kilocalories/kg/day. Preliminary studies in the dog given dinitrophenol indicate that an approximate doubling of the metabolic rate can be obtained but no change in total red cell volume was obtained after 84 days indicating that at least in this animal oxygen consumption is not related to erythropoiesis when oxygen consumption was increased in this manner. In the dog made hypothyroid by radioiodine, the metabolic rate is approximately one-half normal. In these animals there is a corresponding decrease in the total red cell volume by half. Replacement therapy with thyroid indicates that the metabolic rate and total red cell volume can be restored to the normal state.

DERMATOLOGY BRANCH

The clinical and laboratory research program of the Dermatology Branch continues to be primarily concerned with three major areas: (1) mycosis fungoides lymphoma; (2) epidermal growth and differentiation; and (3) pteridine metabolism and pigmentation.

Mycosis Fungoides Lymphoma

The seemingly fundamental relationship between immunologic reactivity of patients with this disease with the development and course of the disease prompts a continuation and intensification of efforts to explore such a relationship. The disease ordinarily begins with a reactive cutaneous dermatosis of eczematous type, which may persist for long periods prior to the appearance of a disease process recognizable as a lymphoma. Some studies have been directed to patients in this early stage of development, such as evaluating their general immunologic reactivity. Although these patients are found to be within the range of

normal in being capable of reacting immunologically to various stimuli, the degree of reactivity compared to normals has not been established. Autologous transplantation and implantation of skin involved with early stages of disease has failed to show that the disease is transplantable at this stage.

Several areas require extended exploration. Among these is the question of how to recognize malignant (neoplastic) lympho-reticular cells in an early stage infiltrate, composed of normal appearing cells for the most part. No morphological or behavioral characteristic is known that would help identify such cells. Early lesions injected with tritiated thymidine reveal that many cells, morphologically resembling both benign and malignant cells, synthesize DNA and hence undergo mitotic division. The ability of cells from such an infiltrate to react *in vitro* to immunologic challenge hopefully will provide essential data in this regard. The addition of an electron microscopic facility into the research program hopefully will yield important information on morphologic recognition of early malignant cells.

An encouraging, but yet unexplained phenomenon, is the observation made in several patients to date that dinitrochlorobenzene (DNCB) applied to early lesions of mycosis fungoides causes the lesion to clinically disappear. Whether this event is due to a hypersensitivity reaction or to a direct chemical effect has not yet been established, but studies to settle the question have been initiated.

Epidermal Growth and Differentiation

The governing relationship, other than a simple nutritive one, between epithelial cell populations normally and in neoplasia remains an area under active exploration. The fact that epithelial cells in tissue culture, under the highly artificial conditions of defined chemical media, readily depart from the normal both morphologically and functionally indicate that normalizing factors in the native environment is important in carcinogenesis, and perhaps in the course of the neoplastic process. Also, the fact that successful therapy of some types of epithelial tumors is not in itself cytoidal suggests that alterations of environmental condi-

tions are operative in altering the growth and course of such tumors. The known therapeutic effect of topically applied 5-fluorouracil is being examined for its possible mode of action on keratoses (pre-malignant lesions) and basal cell tumors. Specific alterations of the physical environment of some lesions are also being performed to assess how these modify tumors such as superficial basal cell epitheliomas and keratoacanthomas.

Pteridine Metabolism and Pigmentation

In the course of study on the mechanism whereby a known microorganism, utilized for the purpose, converts tyrosine and coumarin to a pigment, attention was directed to the hydroxylating (oxidative) systems involved.

The finding of two, and most likely three, different hydroxylating systems in this organism currently under study offers a rather unique opportunity for studying the mechanism(s) of action of enzymatic hydroxylation. All three systems have characteristics in common and yet sufficient differences to make a comparative study quite revealing. It is hoped that study of the common cofactor requirement for a flavin (FAD) will reveal the series of events occurring on the enzyme surface during hydroxylation.

DIAGNOSTIC RESEARCH BRANCH

Office of the Chief

Very brief summations of the activities of the Chief of the Diagnostic Research Branch are included in the two so-called "projects" that follow. Each is a compilation of several activities which could, undoubtedly, be presented as numerous individual projects. For example, the first project reported on has three sections and involves active association with over nine universities as Project Officer, and the review of over 2400 cytological preparations (early bladder cancer diagnosis and diagnosis of tumor cells in the peripheral blood), the development of a new instrument (CYDAC), daily discussion with one or more of the contractors, and attention to administrative details involved in contract work.

The second so-called "project" involves collaborative activities on a part time basis with over 12 investigators. Much of the work on L₂C leukemia, although very necessary, has provided, unfortunately, essentially negative experience. The few positive leads have been described under "Major Findings." It can be speculated that additional work could have been accomplished were more guinea pigs available. With current techniques, the cost in animals for transplantation alone has prevented the construction of protocols which could lead to anything but preliminary interpretation.

Finally, not emphasized in the project reports are supportive activities of the Chief of the Diagnostic Research Branch. These included:

(1) Function as Guest Editor for *Acta Cytologica* in the development and compilation of the two issues on the Symposium on Tumor Cells in the Peripheral Blood. (*Acta Cytologica*, 9: No. 1 and No. 2, pp. 1-188, 2965)

(2) Histopathological diagnosis of all slides prepared from the experiment on the effect of diethylnitrosamine on guinea pigs and rabbits (a by-product of an experiment undertaken by Dr. Rapp), and the preparation of the final manuscript which appeared in the *Journal of the National Cancer Institute*, 34: 4, April 1965.

(3) The enzymatic analysis of leucine amino peptidase on the serum of monkeys given carcinogens by Dr. Margaret K. O'Gara and Dr. Roger O'Gara, undertaken as a possible means of early diagnosis of liver carcinoma. An abstract was presented at the meeting of the Federation of American Societies for Experimental Biology in April by Dr. Roger O'Gara.

Presumably, these latter two will have been reported elsewhere.

ENDOCRINOLOGY BRANCH

The Endocrinology Branch continues to represent a group of independently active investigators who effectively pool their clinical and experimental skills for the advancement of their respective studies as well as for the maintenance of a high standard of medical care. The blending of these skills provides a favorable

climate for the development of the Branch's varied research efforts.

Further extension of our therapeutic efforts in the field of trophoblastic disease now provides a practical basis for the virtual eradication of the advanced forms of choriocarcinoma initially brought under study. By the proper application of our current diagnostic and therapeutic procedures to all pregnant women almost all women can expect to avoid extensive trophoblastic disease without surgical intervention. Moreover, very few, if any, women should henceforth find it necessary to lose their reproductive function by hysterectomy in an attempt to eliminate early trophoblastic disease.

Our growing knowledge of the complexities of steroid hormone metabolism in normal and in pathological states is the product of increasing sophistication in analytical methodology, the main elements of which are the use of tracer methods and gas chromatography. This is a vivid example of how great an impetus is sometimes provided a field of investigation by the development of new tools. The enormous advances in our understanding that now attend the quantitatively reliable determination of hormone secretion rates and production rates as opposed to the old urinary excretion studies are evident from the reports from our steroid group.

The molecular approach to disease analysis is complemented by the newer developments in chromosomal analysis. These innovations have illuminated many endocrinological syndromes and particularly those related to gonadal abnormalities. The genetic relationships of these syndromes with each other and with other forms of hereditary abnormality of numerous somatic systems have become a matter of prime interest to the modern endocrinologist. Our group's firm orientation in the theory and methodology in this field represents a major contribution to our program.

The endocrinologist must also now be an immunologist of sorts. Our group has participated effectively in the development of new and stimulating techniques for the immunoassay of the pituitary hormones as well as in a further characterization of these hormones as antigens. These observations provide a challenging op-

pportunity for the further exploration of these techniques in the study of normal and abnormal endocrine states.

Extended observations make it increasingly clear that much that has been learned about reproductive and endocrine physiology in the rodent has little or no applicability to man or to the higher primates. Notwithstanding the great logistic burden imposed by work with monkeys, we have pursued an active study of menstrual function and endocrine aspects of pregnancy in the monkey. The dispensability of the ovaries from early pregnancy in the monkey is in direct contrast to the situation in the rat. Hence much of our ideas of therapy of abnormal uterine function in women based on rodent studies will have to be revised.

Neuro-endocrine studies are facilitated through the technique of ectopic transplantation of endocrine organs. Our observations establish that neuro-endocrine relationships are subject to experimental modification through prolonged alteration in the endocrine status of the animal as a whole. These data relate directly to phenomena of aging with its attendant endocrine and oncologic changes.

Administratively, our group appears to have evolved a modus operandi which is at once congenial and effectual. The operating principle is that of least and therefore, we hope, best government. The senior staff functions on an alternating basis in such a way as to provide each member with maximum continuity of opportunity for sustained research effort. This in turn provides ample opportunity for the guidance and stimulation of the clinical and research efforts of the junior staff. The latter's enthusiastic participation in the Branch's work is again reflected this year by an early request for a third year by one of our first year men. However, our second year group this year yielded no candidates for a third year, largely because of prior commitments elsewhere.

Unfortunately, limitations of space and facilities preclude our acceptance of more Visiting Scientists notwithstanding increasingly numerous requests for such opportunities with our group. This may be regarded as a major deficiency in our program since we do profit so much from the stimulus provided by the pres-

ence of such individuals during their stay among us.

It may be anticipated that the coming year will provide a unique opportunity for the more complete exploration of many of the methodological developments which have characterized this year's work.

It is always a pleasure to indicate our warm appreciation for the sustained moral and material support extended the Endocrinology Branch by NCI.

IMMUNOLOGY BRANCH

The Immunology Branch was established less than a year ago from components of the Metabolism Service and the Diagnostic Research Branch. It consists of 5 senior investigators and three clinical associates, plus supporting personnel. In the past year, new laboratories have been outfitted in Building 10, and the Immunochemistry Section moved from the Auburn Building in Bethesda to Building 10 in January, 1965.

Research in the past year has been concerned with molecular and biologic aspects of immunity and largely has been an extension of previous interests of the principal investigators. New lines of work with antigens of carcinogen induced tumors in animals and cellular (leukocyte) antigens in man have begun and show considerable promise for fruitful development.

A notable success was the discovery of a new class of human immunoglobulin, termed immunoglobulin D, IgD, or γ_d , by Dr. David Rowe, a guest worker from Birmingham, England, and Dr. Fahey. This is the first new class of immunoglobulins discovered in man since 1956, and developed directly from studies of the abnormal proteins that occur in multiple myeloma. With the discovery of IgD, the total number of immunoglobulin classes recognized in man are four, these components being IgG, IgA, IgM and IgD (Ig = immunoglobulin).

The new component, IgD, is present in serum of most normal subjects and constitutes less than 1% of the total immunoglobulins. This component can be formed in malignant plasma cells, and at least five cases of multiple myeloma producing abnormal D-myeloma proteins have been identified. The role of the IgD

immunoglobulin in immunity remains to be determined. Immunological studies and studies of the changes of this immunoglobulin in disease are under investigation at the present time. The possible linkage of the structural gene for the δ polypeptide chain, the characteristic chain of IgD, to any of the genes controlling other immunoglobulins also remains to be determined.

Studies of human immunoglobulins were extended by the identification of four subclasses of the IgG immunoglobulins ($7S_{\gamma_2}$ -globulins) by Dr. W. Terry. These components, tentatively termed γ_2a , γ_2b , γ_2c and γ_2d , are present in normal serum as well as among G-myeloma proteins. These subclasses probably differ functionally, and Dr. Terry has shown that there are differences in skin-binding activity between these four classes of immunoglobulins. He is actively extending this work now and has preliminary data indicating differences in the genes controlling these subclasses of immunoglobulin.

Investigation in mice also revealed a new class of immunoglobulins and new subclasses of IgG or $7S_{\gamma}$ -globulin. Five immunoglobulin forms are now recognized in the mouse: IgM, IgA, $7S_{\gamma_1}$, $7S_{\gamma_2a}$ - and $7S_{\gamma_2b}$ -globulin. The 7S immunoglobulins were found to differ in genetic control and in functional activity in terms of the skin-sensitizing ability in homologous and heterologous species, and in response to immunization.

These findings in man and mouse indicate an extensive heterogeneity within the immunoglobulin family, but also indicate an order to the levels of heterogeneity. Appreciation of the various levels of heterogeneity will facilitate correlations between structural genes and specific polypeptide chains and between structure and function in the immunoglobulin molecules. Efforts will be expended in the coming year to identify specific amino acid sequences, carbohydrate content and other features of the immunoglobulin molecule which are characteristic of specific antibodies and of molecules with particular genetic factors and immunologic functions, such as complement fixation, skin fixation or transfer across the neonatal intestinal tract, etc.

Studies in various conditions of immunization in mice revealed that specific immunoglob-

ulins were affected differently depending on the mode of administration and the presence or absence of adjuvant. This has profound biologic effects, because not all antibodies have the same functional characteristics. There also seem to be genetic differences, which indicate that genetic factors control immune response.

The possibility of genes controlling immune response was investigated in man by extension of studies in a patient who has selective immunoglobulin deficiency. This patient has a very profound deficiency of IgG and IgA immunoglobulins, but has a superabundance of IgM and IgD. An extensive series of immunologic, physiochemical, cytologic, biosynthetic, immunofluorescent, electron microscopic and metabolic studies indicated that this patient was able to produce the particular polypeptide chains unique for IgG and IgA only in very small amounts. A defect in the regulatory process, very likely in a regulatory gene(s) controlling the synthesis of these two polypeptide chains (and, hence, of the IgG and IgD forms of immunoglobulin molecules), is the probable basis for this abnormality. Further studies of this phenomena are indicated with patients who have other forms of immunoglobulin disorders, because investigation of the regulatory defects will probably provide considerable insight into the exact mechanisms by which immunoglobulins are formed.

The mechanisms of immunoglobulin formation are just beginning to be investigated. It is hoped that *in vitro* studies with isolated systems as well as appropriate *in vivo* investigations will illuminate the processes by which antigen recognition occurs, antibody response is initiated and immunoglobulin formation is accelerated in the normal state, as well as in disease.

Immunoglobulins, once synthesized, depend upon their physical properties to determine their length of survival. The macroglobulins (IgM) for example, are catabolized at four or five times the rate of the 7S IgG antibodies. Recent studies conducted with Dr. Sell have shown that even the IgG molecules are heterogeneous in their catabolic properties. These studies were carried out in mice and showed that three subclasses of IgG differ in their catabolic properties, and their half-times may

differ by as much as a factor of two. This is an important factor in determining survival of antibodies once formed, and strongly influences serum level of individual antibodies. Mouse macro- and mouse IgA globulins apparently have no homeostatic regulatory mechanism to help control the level of those molecules. The catabolism of these molecules, however, is much faster than of the IgG group.

Catabolic studies will be extended to the IgD molecules and to the subclasses of human IgG immunoglobulins in the coming year. It seems probable that the normal human IgG immunoglobulins differ in their catabolic properties because heterogeneity has been observed in the catabolism of normal IgG in previous studies in our own and other clinics.

Immunochemical technics for the diagnosis of multiple myeloma and macroglobulinemia were extended to making available reagents for discerning Type I and Type II immunoglobulins. These are differences from the IgG, IgA, IgM and IgD classes noted above, and are based on differences in the light (L) polypeptide chain properties. Reagents were supplied to Dr. Bergsagel, who has found that patients with Type I Bence Jones protein respond much better to chemotherapy with melphelan than patients with Type II Bence Jones proteins. This initial observation will have to be extended and checked by other groups conducting chemotherapy studies. It also raises the question of whether the different forms of heavy chain noted above in the subclasses of IgG will make a difference in clinical course or the response to therapy of tumors forming these types of protein.

Conduct of such studies will require more reagents and greater facilities than are available within the Immunology Branch of the NCI. Because of this need arrangements have been made to have some of this work carried out by a suitable contractor. It is hoped, by means of this contract, to extend the facilities which are now available in only a few research laboratories to all of the clinics participating in the Cancer Chemotherapy Cooperative Studies of multiple myeloma and related diseases. Particular emphasis will be upon a classification of the abnormal proteins and the relationship between the type of abnormal protein and the

prognosis and response to therapy and other clinical features of malignant disease. The diagnostic facilities also will be extremely valuable in recognition of macroglobulinemic lymphoma, and also should assist in the recognition of agammaglobulinemia and other immunoglobulin deficiency syndromes.

Clinical investigations in Waldenström's macroglobulinemia were continued with confirmation of the value of plasmapheresis in reducing hyperviscosity symptomatology. It is anticipated that future work will be concerned with the distinctive cells occurring in chronic lymphocytic leugemia, Hodgkin's Disease, macroglobulinemic lymphoma, plasmacytomas and related malignant diseases. In order to carry out these studies, facilities have been established within the Immunology Branch, as well as in cooperation with the Medicine Branch, for characterizing the cellular antigens of leukocytes, with particular emphasis on the lymphocytes and plasma cells. Studies have been initiated, both in mice and in man, for detecting the known cell antigens, as well as procedures for identifying new antigens, which can be related to the malignant process or to the normal functional features of these various forms of cells.

The Immunochemistry Section of the Immunology Branch has been conducting studies on the antigenicity of carcinogen-induced tumors in guinea pigs and rabbits. Quantitative study of the antigens of hepatomas induced by nitrosamine, compared with liver from the same animal prior to treatment, as well as with normal liver obtained at the time the tumor was removed and liver from other animals of the same strain has been an important aspect of this work by Dr. Rapp and associates. Much more Forssman antigen was found in the tumor than in normal tissue. Studies are being extended to other antigen detecting systems to see if the differences are wholly quantitative between malignant cells, or whether there are qualitative differences as well.

A new test for antigen-antibody reactions, which has great sensitivity as well as precision, has been developed by Drs. Borsos and Rapp. This test is termed the C'1_a-fixation and transfer test. By using their knowledge of the procedures of the mechanisms of complement

activation, these investigators developed the new test, which should be exceedingly useful in studies of cytotoxic reactions *in vitro* as well as in the nature of the antibodies and cellular antigens required in this process. This is a very active program which should be extremely useful to many of the investigators in the NCI involved with the immunology of neoplasms.

Clinical studies will develop in the direction of efforts to find immunochemical uniqueness of neoplastic cells, as well as the specific effect of malignant disease on the immune process. We hope to make some further investigations into the suppression of antibody formation that occurs in chronic lymphocytic leukemia, as well as the suppression of delayed hypersensitivity that occurs in Hodgkin's disease, by means of studying isolated cells or tissues from patients with these diseases.

Studies of transplantation immunity in the Immunology Branch will be concerned with transfer of lymphocytes and other immune active cells. Collaboration with other Branches concerned with transplantation of other cell lines or organ systems is being developed. We now have a capacity for detecting the genetic determinants of human immunoglobulins, *i.e.* the Gm and Inv factors. This capacity should help in selection of donor-recipient transplantation combinations, as well as in efforts to determine the success of immune cell transfer. The systems for detecting cell antigens now under development also will be helpful in this work.

Efforts will be made to selectively suppress immune response so that cell and tissue transplantation may take place without the hazards of bacterial infection, *etc.* that result from general suppression of immunity.

Finally, efforts will be directed toward preparation of cell fractions with immunologic information. Some of these are expected to make antigens more potent and, hopefully, others may permit sensitization against specific foreign antigens.

RADIATION BRANCH

During the past year, the clinical and experimental research activities of the Radiation Branch have been redeveloped to complement

its continuing service functions. This report will review the programs which have been undertaken and will describe the objectives of projects being established.

A major effort was the comprehensive appraisal and reorganization of all radiotherapeutic procedures as a prelude to the initiation of new clinical research studies. During this period, treatment techniques have been standardized, dosimetry planning procedures have been established for reproducibility from patient to patient, and routines have been instituted for the undertaking of clinical investigations.

Following this reorientation toward an increased emphasis on clinical research, a number of studies were undertaken. Primary in terms of patient accession numbers has been the evaluation of radiotherapy in Hodgkin's disease. The value of intensive radiotherapy in localized Hodgkin's disease has been generally accepted, and the Radiation Branch has undertaken studies to investigate the role of radical radiotherapy in advanced Hodgkin's disease as well as the evaluation of extended radiotherapy in localized cases. Another area of intense interest is the use of total body irradiation in the chronic leukemias and disseminated lymphomas. Although the number of patients admitted to the total body irradiation studies is as yet small, some very promising results have been obtained in terms of clinical remissions.

A pilot study combining radiotherapy and chemotherapy has been undertaken on Ewing's sarcoma. Two patients have received treatment and both are clinically well for periods exceeding the median duration of remission for this disease. Preliminary studies evaluating increased oxygen tension at atmospheric pressure for potential radiation enhancement have commenced as have controlled clinical radiobiological investigations of dose-time relationships.

A cooperative study on endometrial carcinoma has been organized by Dr. John Marshall of the Surgery Branch, NCI. The Radiation Branch will serve as the radiotherapy center for this study, and will review the radiotherapy of all patients entered into the study by the participating institutions. The Acting Chief of the Radiation Branch serves as Chairman of

the Radiotherapy Subcommittee for the Cooperative Study Group.

A scanning device for the measurement of the transmission thickness of patients has been developed by the Physics Section of the Radiation Branch. The clinical application of this unit is underway and shows considerable promise of increasing the accuracy of dosimetric calculations rendered difficult by body density inhomogeneities.

Several laboratory research projects related to the chemotherapeutic and radiotherapeutic effects on mammalian tumor cells have been completed. The L1210 murine leukemia system has been employed and biological and cytogenetic alterations induced in tumor cell populations as a consequence of treatment have been investigated. Alkylating and antimetabolite drugs as well as ionizing radiations have been found capable of altering the growth kinetics of L1210 leukemia. The effect of these changes on sensitivity to treatment and attempts to correlate these findings with clinical observations are currently receiving attention. Other experimental studies using total body irradiation are designed to pursue a possible relationship between tumor response and dosage-fractionation schedule in order to establish a model approach as the basis for systematic clinical trials of this therapeutic modality.

A number of studies in collaboration with other areas have proceeded during the year. These include an evaluation of pre-operative radiotherapy in carcinomas of the upper air passages (and in other selected instances) with the Surgery Branch, NCI, the investigation of combined chemotherapy and radiotherapy in Hodgkin's disease with the Medicine Branch, NCI, and the continuing assessment of pituitary irradiation for acromegaly with the National Institute of Arthritis and Metabolic Diseases. The considerable clinical and non-clinical irradiation services, including consultative services, have been a continuing responsibility of the Radiation Branch.

The expanding program of research has brought to the surface the acute need for the renovation of the physical facilities for both experimental and clinical purposes. The excessive amount of time required by professional staff for maintenance, combined with the rela-

tive inflexibility of the present machines, are limitations to the research productivity of the Branch. The replacement of the outdated equipment with modern, flexible units is under consideration at the present time.

SURGERY BRANCH

The Surgery Branch of the National Cancer Institute continues to act in a dual capacity, that of carrying out its own specific clinical and laboratory investigations and that of providing consultation surgical service to other branches of the Cancer Institute and to the clinical units of each of the other institutes of the National Institutes of Health. This service includes not only general surgical assistance, but urological, otolaryngological, gynecological and a portion of the thoracic consultations necessitated by the broad scope of investigative clinic studies being carried out by the various institutes.

There were 931 individual consultation requests answered by the Surgery staff, 306 on NCI patients, and 625 on patients from other institutes. 471 operative procedures were required in carrying out these consultations, 215 on NCI patients and 256 on patients from other institutes, 27% of which entailed a major surgical procedure. In addition, 356 surgical procedures were carried out on patients admitted to the Surgical Branch, 58% of which were major operations. A total then of 895 surgical procedures have been performed during the 12 months covered by this report. The entire surgical consultation service was augmented by consultants from the community who saw 247 patients, 233 of which were non-malignant otolaryngological problems. These consultants performed or assisted in 21 surgical procedures.

The surgical Branch has not tried to direct its clinical interests in any one anatomical field, but has studied patients as they have been referred to the branch. A rather aggressive surgical approach to cancer has been carried out, in great part dictated by the fact that over 70% of the patients undergoing cancer surgery have been treatment failures before their referral and admission to N.I.H.

Malignant disease of the head and neck areas continues to be an anatomical site of prime interest to the Surgery Branch. The double blind preoperative radiation study of head and neck cancer patients now include 47 patients. One thousand roentgens given to the tumor area 24 hours preoperatively has caused some increase in morbidity, but the followup time is too short to determine the effect the radiation may have on the end results. The study calls for untreated patients; for this reason numbers are accumulated very slowly.

Survival by conventional means of therapy from paranasal sinus cancer is very poor due to tumor extension into the ethmoid, sphenoid and pterygoid areas, resulting in local recurrence in spite of the usual surgical or radiological techniques of treatment. 31 patients have now had a combined intracranial, transfacial en bloc resection of the paranasal sinus area, including the cribriform plate and medial orbital walls, usually leaving the orbital contents intact. Preliminary followup suggests that with no increase in operative mortality, this aggressive procedure is resulting in an increased number of patients being given a chance of cure. An increased two year survival is now evident.

Admission surveys of patients admitted with oral, pharyngeal and laryngeal cancer continues to show a significant correlation between smoking and alcoholic intake and the anatomical site of their tumor and of the incidence of multiple primary head and neck malignancies. The morbidity and mortality related to the surgical exploration of these advanced cancers has been significantly lessened by the increased attention that has been directed toward better selection of skin incisions, the use of antibiotic prophylaxis and the use of the cervical esophagostomy as a convenient and well-tolerated mode for prolonged tube feeding and keeping food away from healing suture lines. Of 16 patients who have had 19 spontaneous ruptures of the carotid artery during postoperative convalescence over the past 10 years, only 4 such occurrences have developed since 1961. This has in great part been due to the attention which has been directed to the above considerations. Alertness on the part of the hospital staff has provided control for 17 of

these hemorrhages. 16 have been restored without complication to their pre-rupture status.

Patients with carcinoma of the cervix continue to be referred, having had previous definitive surgery, supracervical hysterectomy, inadequate irradiation, and histories of never having had a Papanicolaou smear taken. We are convinced that this disease, which now ranks second in prevalence of cancers in women, could in great part be controlled by either radiation or surgery if the disease was recognized in its earlier stages. This definitely can be done by the routine Papanicolaou smear technique. Our advanced stage of disease patients, with carcinoma of the cervix, show an over all 43% five-year survival following extensive surgery for advanced, usually recurrent disease and an operative mortality of less than 10%. The patients who underwent radical hysterectomy have a 70% survival, anterior pelvic exenteration, 34%; and total pelvic exenteration, 24%.

As we are developing better techniques of selection of patients with advanced pelvic disease by aggressive surgery, the survival following radical surgery continues to improve. Unilateral leg edema, sciatic distribution of pain, bilateral renal involvement, lymphangiography, renal and surgical physiological studies, all are clinical and diagnostic aids in determining feasibility of cure. For advanced tumors of the pelvic organs; vagina, cervix, uterus, bladder and rectum, surgery continues to offer the best chance of cure but this is not done without, at times, severe morbidity. Improved techniques of blood volume determinations, red cell survival studies, bowel sterilization techniques, renal and surgical physiological studies through isotope renography, vital sign monitoring and accurate fluid and chemical replacement determinations all combine to make exenterative surgery a practical and potent means of bringing under control advanced pelvic malignant disease.

A study on preoperative bowel sterilization to determine the efficacy of a standard 3 day bowel sterilization previous to intestinal surgery has confirmed that 5 days of preparation is not necessary. Equivalent doses of Kanamycin and Paromomycin have been satisfactory in

reducing postoperative complications and in 70 patients, no instance of staphylococcal enterocolitis has been encountered.

Lymphangiography, while an aid in preoperative studies, finds its greatest value in a guide for more complete lymph node dissection at the time of surgery. This procedure remains a routine in the preoperative evaluation of all admissions with pelvic disease.

The use of the ileal conduit as a mode of urinary diversion continues to be the most satisfactory means of diverting the urinary stream following bladder removal. This technique of diversion both in short and long term studies results in few complications and preserves excellent renal function in nearly all instances, if attention is directed toward antibacterial prophylaxis in preventing infectious complications. Of interest has been a review of 13 patients who have undergone urinary diversion in spite of preoperative and confirmed operative evidence of one ureter being completely blocked by tumor. Diverting this ureter, after high transection, into an ileal conduit, has resulted in all but 1 of the kidneys returning to function, half very satisfactorily and the others with a variable degree of impairment. Plastic reconstruction of a vagina in selected cases, intensive colostomy and urinary ileostomy instruction and guidance in patients who have had radical pelvic surgery can and does result in a normal, pleasant and productive life.

During the past year, 9 patients with malignant melanoma have undergone 17 major surgical procedures in an attempt to eradicate their disease by wide local resection of the primary tumor and incontinuity lymph node drainage area resection. An analysis of 35 patients operated on more than 5 years ago for locally recurrent melanoma reveals only 8 still alive and free of recognized disease. 13 survived more than 5 years while only 4 survived more than 10 years. It has become apparent that lesions of the scalp and face are prone to develop lymph node metastases in the parotid area and superficial parotidectomy is mandatory if node dissection is to be carried out. It is also apparent that our survival results are most discouraging and with this in mind, several avenues of study are underway both in the

experimental animal laboratory and in the clinic, such as intralymphatic administration of therapeutic radioisotopes or combined chemotherapy infusions or perfusions, with potential techniques of bone marrow protection.

An increasing number of extremity sarcomas, all recurrent following previous treatment, are being seen by the Surgery Branch. Selected instances of amputation and particularly hemipelvectomy, which allows removal of all extremity muscular and fascia, has been performed with no mortality and good physical rehabilitation.

A long term survey of infectious complications following cancer surgery shows that the patient who carries a pathogenic organism with him into the operating room, whether it be from the skin, nose or throat or particularly within the tumor, runs a significantly increased risk of developing an infectious complication postoperatively. Staphylococcal infections predominate but are more easily controlled and cause less patient morbidity than other usual hospital organisms. Antibiotic therapy remains a routine with all patients undergoing cancer surgery, Chloramphenicol being the most satisfactory and frequently used drug. This very potent antibiotic has been extensively used for five years by the Surgery Branch, without evidence of hematological complications. By employed high doses for a limited ten day interval, infectious complications over the past year have been 7% as compared to 22-30% with no antibiotic therapy.

A combined laboratory and clinical program is underway in an effort to recognize, explain and quantitate a few of the many physiological parameters which change following extensive cancer surgery. Accurate and precise methods of measuring the total red cell volume, plasma volume, and extracellular fluid volume have thus far been developed and are currently in use. In addition, the neuroendocrine mechanisms controlling aldosterone secretion are being studied and an attempt to develop an immuno-assay procedure for the determination of circulating anti-diuretic hormone is being made.

In a search for the culpable circulatory defect in refractory shock, all the usual hemodynamic and biochemical parameters thought to be

important in shock are being studied, plus several methods previously unused in this setting. These latter include radioisotope clearance methods for measuring capillary blood flow, electrocapacitance plethysmography and indicator dilution methods for measuring regional blood flow and distribution of cardiac output and dextran molecular weight dispersion measurements as indicators of altered capillary permeability. The use of experimental animal models in the LD₅₀ range and serial studies on patients undergoing extensive radical surgery should provide better information about the early changes developing in those instances where refractory shock eventually occurs as compared with the subjects in which refractory shock does not develop.

During the past year, little attention has been directed toward further refinements in techniques of isolating tumor cells from the blood of cancer patients. It remains apparent that if enough blood is sampled from the cancer-bearing patient, tumor cells will eventually be isolated. Surprisingly enough little correlation has been noted between patient survival and the presence or absence of circulating tumor cells. Tumor cell isolation, identification and interpretation of significance, from wound washings and drainages has similarly been unrewarding in most respects. Possibly due to better techniques of filtering and certainly due to better criteria of identification of individual cells by the cytologists, the incidence of tumor cells identification has decreased to such a low percentage cure as compared to previous years, that validity of the over-all study is questioned. We are, however, using proflavine hemisulfate, an acridine dye, as a tumorcidal wound washing agent in a continuing clinical study.

A comprehensive study of the human thoracic duct lymph has been undertaken by the Surgery Branch. Suppression of the human immune response has been demonstrated with drainage of lymph and further studies are in process in an attempt to elucidate the active substance in lymph which inhibits man's ability to form antibodies when challenged by a primary antigen and prevents normal rejection of skin homografts. Lymphocyte depletion has not been the answer in that immune suppression does not occur when cell-free lymph is

returned to the patient. Studies are in progress to determine the importance of serum immune globulins in this phenomenon. Recirculation of lymphocytes from the blood through lymphatic filters and then through the thoracic duct back into the systemic blood is being studied by means of radioactive tags on the cells and by chromosome markers. Storage of lymphocytes is also being studied by means of vital stains and cell transformation in an attempt to learn more about the life span of human lymphocytes. Production of immune globulins by thoracic duct lymphocytes, in contrast with serum lymphocytes, is also underway. This method of human immune suppression has significance in organ transplantation since it has been shown to be well tolerated by man. Further investigative work both in animals and in humans, is underway to determine the value of this method of immunologic suppression.

Lesions of the renal vasculature causing hypertension have been of major interest to the Surgery Branch with studies directed toward improved diagnosis and surgical management. The isotope renogram is used as a screening test for renal disease, and is valuable for following changes in renal function after revascularization procedures. Differential function studies supported by renal arteriography remain the basic diagnostic procedures. After the Stamey test (during which urinary oxygen tension is monitored), a cardiac phonocatheter is passed transureterally to each renal pelvis where sonic vibrations of the renal artery pulse are recorded. These ancillary diagnostic procedures provide new evidence regarding the renal hemodynamic and metabolic changes during ischemia.

Laser energy has been established as an oncolytic agent for a wide variety of experimental tumor systems. A technique has been developed which permits the quantitative destruction of malignant tissue by laser energy. The destructive capability of laser energy is dependent on the particular wavelength absorption of a given tumor. A technique to correlate the wavelength absorption properties of experimental animal tumors and human tumors has been assessed. Pulsed laser energy can be used to destroy effectively, rapidly, precisely, and safely

multiple malignant tumor implants. Very high energy laser impacts to the intact canine head have produced only superficial localized burns with no untoward effects. It appears that laser energy may be a potential adjunct to cancer therapy.

The cooperative, prospective study of the primary therapy of endometrial carcinoma should provide an answer to an old problem. The different types of therapy under study are surgery alone, preoperative radiation and surgery, and surgery and postoperative radiation. Patients will be assigned to therapy at random. Utilizing the Surgery Branch, as the Study Center, data from cooperating institutions will be collected and analyzed. The study is planned to include 400 patients. Ultimate analysis of the data after the completion of the five year followup will provide meaningful information concerning the relative effectiveness of the methods of therapy under study.

Diagnostic culdoscopy, gynecography, exploratory laparotomy, culdoscopic photography and culdoscopic ovarian biopsy have all been used to compare the usefulness of culdoscopy and gynecography, to develop a technique of more exact and dynamic culdoscopy and to compare the usefulness of the various culdoscopic instruments available. Culdoscopy and gynecography have been compared in 44 patients and the accuracy and capabilities of each technic noted. An intraperitoneal calibrated probe has been developed which permits manipulation, palpitation and accurate mensuration of the pelvic organs. Techniques of photography and ovarian biopsy have been utilized to give permanent gross and microscopic specimens of the ovaries.

Pergonal (human menopausal urinary gonadotropin) is being utilized as an ovarian stimulatory agent in a development of an ovarian stimulation test. 24-hour urinary estrogens, testosterone production rates and delta 4 androstine-dion secretory rates are being measured. This test will prove useful in the diagnosis of amenorrhea and in the study of ovarian function. However, it will also be of value in the diagnosis and study of endocrinologically active ovarian neoplasms.

Induction of ovulation in anovulatory women with Pergonal and human chorionic gonadotro-

pins has been possible but has been relatively unsatisfactory because of the 40% of multiple pregnancy rate. Utilizing patients studied as a part of the ovarian stimulation test studies, dosage regimens of these two agents are being evaluated. The objective of the study is the selection of dosage regimes most effective for induction of consistent, single ovulations in anovulatory women.

Urine oxygen tension (pO_2) has been studied in dogs under standard conditions of anesthesia, temperature, and urine sampling in both hydropenia and water diuresis. Osmotic diuresis in hydropenia and free water clearance uniformly drops pO_2 . This suggests that pO_2 is related to sodium reabsorption in the loop of Henle, i.e., that increased sodium reabsorption leads to increased oxygen consumption in the renal medulla, and therefore, to a fall in urine pO_2 . Studies with ethacrynic acid which blocks the sodium pump in the loop of Henle would appear to support this theory.

In various experiments, rats and guinea pigs have been transplanted with excessive numbers of histocompatible endocrine tissues in order (1) to study the ability of normal tissue to reduce its synthetic activity and (2) to magnify small physiological effects of normal amounts of transplanted tissue and thus clearly define their activity. By using excessive numbers of transplanted isologous parathyroid glands, we have found that a state of obligatory hyperparathyroidism results. On the other hand, by using up to 30 transplanted pituitaries in a single hypophysectomized recipient, we have been able to demonstrate trophic activity from transplanted tissue proportional to the dose transplanted and much higher than previously observed. Similarly, 10 to 20 transplanted animals have been demonstrated to reverse the effect of pinealectomy in female rats.

A combination antimetabolite-metabolite program of therapy is being developed to administer systemic chemotherapy for disseminated neoplasms while protecting the anti metabolite. Previous combinations of such drugs have been given and usually in the ratio of 1 to 1 or 2 to 1 with the metabolite being given in very high concentrations intramuscularly or intravenously. Since very little lasting benefit

has been obtained in human neoplasms from such a combination of drugs, it is proposed that the metabolite when given in this high concentration voids or blocks the effect of the antimetabolite on the cancer cell as well as in susceptible tissues such as the marrow and gastrointestinal tract. Because of the slow doubling time of most human neoplasms, prolonged exposure of a cancer cell population to the antimetabolites is a logical approach to chemotherapy. Such an exposure for several weeks may be feasible, utilizing intra-arterial protective specific metabolites on normal rapidly proliferating cell populations susceptible to toxicity. Encouraging toxicity studies in primates have been underway during the past year, protecting the iliac marrow by infusion of the metabolite into the superior gluteal artery. Pilot studies are now proposed which will determine the clinical feasibility of this project.

Ethiodol is a commonly used lymphangiographic radio-opaque medium. Preliminary carcinogenesis studies in mice suggested that this agent might have activity which would discourage its further use. These animal studies utilized Ethiodol. Subsequent animal investigations, comparing the poppy seed oil component of Ethiodol, with Ethiodol itself and other similar agents are now being followed in several strains of mice as well as in rats and dogs. Preliminary data suggests that the poppy seed oil, as previously reported, is carcinogenic in certain mouse strains.

A study of tumor growth and metastases with transplanted, induced and spontaneous tumors in mice has shown that metastases observed in these three separate tumor systems, in both amputated and intact tumor-bearing mice was similar even though the growth rates of the tumor systems varied markedly. The similarity in the extent of metastases is believed to be due to prolonged survival which was permitted by individual animal housing. On the basis of this study, it appears that three tumor systems evaluated might be interchangeably used in metastases studies if recognition is to their varied growth rates.

Induced and spontaneous tumor systems were studied to determine if the rate of tumor growth and the number of metastases would be altered if a second tumor was transplanted into

the host after the initial tumor had been removed. We observed that the tumors implanted after the initial tumor had been removed, grew in exactly the same manner as they did in control animals even when metastases from the metastatic tumor were extensive.

In a controlled study using inbred mice and transplantable tumor systems, incisional biopsy of the tumor was shown to increase significantly the incidence of pulmonary metastases. It was observed in one tumor system that more animals possessed small foci of tumor in their lungs four weeks after their primary tumor was removed than would be expected to die of pulmonary metastases. These foci were infiltrated with mononuclear cells and cells in mitosis, suggesting that the tumor cells were being rejected by the host.

Studies have been carried out to determine the role of the lymphoreticular tissues in chemically-induced tumor immunity. Splenectomy prior to tumor inoculation had no effect on tumor immunity. Splenectomy performed three weeks after tumor inoculation, however, removed most of the animal's resistance to further tumor challenge. Regional lymphadenectomy had the opposite effects. When lymphadenectomy was performed prior to tumor inoculation, host resistance was markedly depressed but when performed after the tumor was growing, no effect on tumor growth was found. Adoptive transfer of chemically-induced tumor immunity was possible when large doses of immune spleen or lymph node cells were given intraperitoneally one week prior to tumor inoculation. Further studies are now underway to investigate whether the adoptively transferred immune cells are competent within diffusion chambers, and whether immune RNA is responsible for the transference of tumor immunity.

Studies have been performed to investigate the nature of the immunity imparted by certain chemically-induced tumors upon their hosts. Particular emphasis has been focused on whether chemically-induced tumor immunity exists in the autochthonous host, and whether the specific immunity that we see in later transplant generations is due to antigenic alteration of the tumor in serial passages. The

cellular localization of this antigen seems to be in the nuclear fraction of the tumor tissue. With weakly antigenic tumors, i.e., S-91 melanoma, prior immunization with cell-free tumor extracts had no significant effect on tumor growth. Enhancement occurred when this extract was given at the time of tumor inoculation.

The Surgery Branch has been pleased to cooperate with the Precious Blood High School of Holyoke, Massachusetts in having five of their students spend time with us in preparation for their student science projects. This work was directed toward experimental metastasis in mouse tumor systems. Instruction, direction and limited equipment and animals were supplied to these students in order that their studies could be completed back at their school. Carrying out separate projects, both in 1963 and 1964, all five have won awards and cash

prizes from their school, the American Cancer Society, and state-wide awards sponsored by Massachusetts Institute of Technology.

Four Clinical Associates received recognition and prizes for their work. Drs. Riggins and Pilch received first prize for their paper on basic studies on the antigenicity of fibrosarcoma, from the American Academy of Orthopaedic Surgeons. Dr. John Minton was given the James Ewing Society's Resident Award for his work on laser radiation on animal tumor systems, and Dr. Ruben Gittes won a first prize from the American Urological Association for his paper on transplanted isologous parathyroid glands as an experimental model for the study of hyperparathyroidism. All of these papers were judged best in their respective groups in competition with surgical residents from medical centers all over the United States.

NATIONAL HEART INSTITUTE

LABORATORY OF BIOCHEMISTRY

Section on Enzymes

Research in this laboratory continues to involve studies on a number of fundamental problems that provide opportunities to elucidate biochemical mechanisms hitherto unexplored. Major areas of investigation include the following: (1) the regulation of divergent biosynthetic pathways of metabolism; (2) the metabolism of one carbon compounds; (3) the metabolism of amino acids; (4) cystathionine synthesis and transsulfuration; (5) the mechanism of action of vitamin B₁₂ coenzyme; (6) the anaerobic hydroxylation of aromatic compounds; (7) the uptake of amino acids by cellular membrane preparations and (8) the dissimilation of heterocyclic compounds. Several of these studies have inevitably embraced broad areas of enzyme function in metabolism such as those concerned with the roles of vitamin B₁₂ derivatives and of non-heme iron electron carrier proteins (i.e., ferredoxin).

The Regulation of Divergent Biosynthetic Pathways of Metabolism

CUMULATIVE FEED-BACK INHIBITION OF GLUTAMINE SYNTHETASE. Previous studies in this laboratory showed that the glutamine synthetase of *Escherichia coli* is uniquely inhibited by eight different metabolites whose synthesis may involve glutamine as a precursor. Whereas the inhibition of glutaminesynthetase activity by any single metabolite is only 10–40 percent, when several or all inhibitors are present simultaneously, an over-all cumulative inhibition is observed that would be expected if each inhibitor is independent in its action and is uninfluenced by the presence of the others. When all eight end metabolites are present simultaneously, over 95 percent inhibition of the enzyme is observed.

In order to determine the mechanism of this unusual type of feed-back regulation, the glutamine synthetase is being studied in detail. After a 200-fold purification from extracts of *E. coli*, the enzyme was isolated as a pure homogeneous protein. It has a molecular weight of approximately 900,000, as estimated by its sedimentation rate in a sucrose density gradient, and it migrates as a single major band during gel electrophoresis. Treatment of the enzyme with 1 M urea and 0.01 M EDTA results in its quantitative disaggregation to enzymatically inactive subunits of approximately 100,000 molecular weight. In the presence of manganous salts the subunits reaggregate to form the native molecule and the enzyme activity is restored.

An explanation for the fact that only partial inhibition is observed at saturating concentrations of each inhibitor was sought through a more detailed kinetic analysis of the reaction catalyzed by the purified enzyme. The data obtained with some of the inhibitors are consistent with the hypothesis that the enzyme contains at least two binding sites for the inhibitors. Binding at one site occurs at relatively low inhibitor concentration and results in inhibition of the enzyme. With increasing inhibitor concentration binding occurs also at a second site (activator site) and results in partial reactivation of the inhibited enzyme, possibly by reducing the affinity of the enzyme for the inhibitor at the inhibitor site.

In view of the central role of glutamine synthetase in nitrogen metabolism and because of its remarkable susceptibility to cumulative feed-back inhibition by a large variety of end metabolites, it provides an excellent model system to study manifold regulation of divergent metabolic pathways. In addition, its high degree of stability and unusual physical properties offer opportunities to investigate several interesting aspects of protein chemistry; i.e.,

the mechanism of its aggregation and disaggregation. Future experiments will be directed at a more intensive investigation of the purified protein in relation to its fine structure and feed-back characteristics.

One Carbon Metabolism

METHANE FERMENTATION. Previous studies in this laboratory have shown that cell-free extracts of *Methanocarcina barkeri* catalyze the reduction of methanol, CO₂ and methyl-B₁₂ to methane. For this reduction, molecular hydrogen serves as the electron donor and ATP and CoA are required. Further studies have shown that cell-free extracts catalyze the accumulation of methyl-B₁₂ when incubated with substrate levels of reduced vitamin B₁₂ (i.e., B_{12s}) methanol, ATP and CoA.

Labeled methyl-B₁₂ has also been isolated after incubation of crude extracts with carbon-labeled methanol and unlabeled methyl-B₁₂ under conditions where both substrates are reduced to methane. However in the latter experiment the amount of labeled carbon trapped in the residual pool of methyl-B₁₂ was considerably less than would be expected if free methyl-B₁₂ is an obligatory intermediate in methane formation. It is concluded that enzyme-bound methyl-B₁₂ or a related derivative, but not free methyl-B₁₂ is probably an intermediate in methane formation.

SYNTHESIS OF ACETATE FROM CO₂. Investigations on the enzymic condensation and reduction of CO₂ to acetate have been continued. The possibility that methyl-B₁₂ is an intermediate in this novel biochemical process was suggested by earlier experiments showing that cell-free extracts of *Clostridium thermoaceticum* catalyze the conversion ¹⁴C-methyl-B₁₂ to methyl labeled acetate in the presence of carbon dioxide, CoA and pyruvate. A role of methyl-B₁₂ in acetate synthesis is further supported by recent experiments showing that ¹⁴C-methyl-B₁₂ can be isolated from incubation mixtures that contained initially ¹⁴CO₂, unlabelled methyl-B₁₂, pyruvate, CoA and crude enzyme preparation. Additional supporting evidence on this point is provided by recent experiments showing that cell-free extracts of *C. thermoaceticum* catalyze the *de novo* synthesis

of methyl-B₁₂ from CO₂ and reduced vitamin B₁₂.

In order to determine the mechanism by which methyl-B₁₂ is converted to acetate, efforts have been made to purify the enzyme system catalyzing this conversion. By chromatography on DEAE cellulose, cell-free extracts were resolved into three protein fractions, all of which are required in combination in order to catalyze the conversion of methyl-B₁₂ to acetate. One of the three protein fractions has been identified as ferredoxin. It was incidentally discovered that extracts of *Clostridium sticklandii* that are unable to catalyze the formation of acetate from CO₂ and methyl-B₁₂, are able to do so if they are supplemented with one of the unidentified protein fractions from *C. thermoaceticum*.

These results indicate that the reduction of CO₂ to acetate is a unique biochemical process that involves pathways of one carbon metabolism hitherto unrecognized.

The Metabolism of Amino Acids

REDUCTIVE DEAMINATION. The reductive deamination of glycine and the fermentation of lysine to form fatty acid derivatives were previously shown to be coupled with the esterification of orthophosphate to form ATP. Various substances, including DPNH, dimercaptans and reduced methyl viologen, are able to serve as electron donors in these reactions, and available evidence indicates that the phosphorylation may be associated with the electron transport process rather than substrate alterations. These reductive deaminations therefore appear to offer unusual opportunities to investigate the mechanisms of anaerobic oxidative phosphorylation in completely soluble enzyme systems. Accordingly, efforts have been made to isolate and purify the enzymes from *Clostridium sticklandii* that are involved in reductive deamination *per se* and also those involved in the anaerobic electron transport chain of this organism.

An acidic, low molecular weight protein component of the glycine reductase system, designated as protein A, has been purified 500 to 700 fold and some of its properties have been studied. Its molecular weight is approximately

20,000. It is moderately heat stable, tends to aggregate with concomitant loss of catalytic activity, and is sensitive to inactivation by exposure to ultraviolet light (360–400 m μ). The latter fact suggests the presence of a light-sensitive chromophore absorbing in the yellow region of the spectrum. Protein A is apparently not involved in the terminal phosphorylation reaction since it catalyzes neither the exchange of orthophosphate with ATP nor of ADP with ATP. It is distinct and is readily separable from the menadione-dependent p-nitrophenyl phosphatase previously discovered in extracts of *C. sticklandii*.

In addition to protein A, five other proteins, appearing to have electron carrier functions have been highly purified from extracts of *C. sticklandii* and partially characterized. One of these is the non-heme iron electron carrier protein, ferredoxin, that is an essential component of the system catalyzing the reductive deamination of glycine and also of the multi-enzyme system catalyzing the reductive cleavage of proline to form Δ -aminovaleric acid. The ferredoxin from *C. sticklandii* is similar in size and acidity to ferredoxins isolated from other organisms and will satisfy the ferredoxin requirements for other enzymatic reactions; i.e., the synthesis of acetate by *C. thermoaceticum* and the reduction of DPN by molecular hydrogen by extracts of *C. kluyveri*.

Of the four other electron-carrier proteins isolated, two are flavoproteins that catalyze the oxidation of reduced pyridine nucleotides. Both are acidic proteins with molecular weights of about 25,000. One of these flavoproteins is a TPNH specific dehydrogenase; the other is a DPNH specific dehydrogenase. They catalyze the transfer of electrons from the respective reduced pyridine nucleotides to oxygen, or any of a number of artificial electron acceptor dyes. Their role in the electron transport system of *C. sticklandii* is indicated by the fact that, in the presence of their respective reduced pyridine nucleotides, they catalyze the reduction of two other electron acceptor proteins that have been partially purified from the cell-free extracts. One of these is characterized by its orange-red color which is lost when it is reduced enzymatically or treated with sodium borohydride or hydrosulfite. It is a small (MW,

6000–7000) acidic protein with a light absorption band having a maximum at 490 m μ . The other electron acceptor protein is red-violet in color, becoming bleached upon enzymic or chemical reduction. It too is a small (MW, ca. 25,000) protein having an absorption band at 535 m μ . The reduced forms of both of the electron carrier proteins are readily autoxidizable and revert to their characteristic oxidized color when exposed to oxygen. Although the precise roles of these proteins are not known, in view of their electron carrier capacities it is reasonable to assume that they are a part of the anaerobic electron transport chain present in this organism. Further studies are aimed at the characterization of their prosthetic groups and the establishment of their biological function.

Incidental to the purification of the above proteins, was the purification also of the quinone-dependent p-nitrophenyl phosphatase. This curious enzyme was previously discovered in extracts of *C. sticklandii* but resisted efforts to purify it by the conventional fractionation procedures then available. It has now been obtained in a more highly purified form as a by-product of the above studies. The mechanism of its action will be re-explored using P³²-labeled p-nitrophenyl phosphate which is now commercially available.

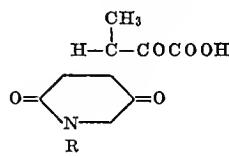
Cystathionine Biosynthesis and Trans-Sulfuration

CYSTATHIONINE γ -SYNTETASE. Cystathione γ -synthetase catalyzes a reaction between L-cysteine and o-succinyl-L-homoserine to form L-cystathionine and succinate. This enzyme has been isolated from *Salmonella* extracts as a pure homogeneous protein. The purified enzyme has a molecular weight of $160,000 \pm 5,000$ and contains 4 moles of pyridoxal phosphate per mole. When treated with guanidine and β -mercaptoethanol at pH 7.0 it is resolved into 4 identical subunits (MW=43,000). Kinetics of the enzymatic reaction have been studied in detail. The Km's for o-succinyl-L-homoserine and L-cysteine are 7×10^{-5} M and 7×10^{-6} M, respectively. The product of the reaction, L-cystathionine, was isolated as a pure crystalline solid and was positively identified by direct comparison with an

authentic sample of L-cystathionine. The enzymatic product and the authentic sample were identical with respect to chromatographic behavior, appearance in light, phase contrast, dark field and polarized light microscopy, infra-red spectra, optical rotary dispersion, chemical reactivity and enzymic activity.

The purified cystathionine- γ -synthetase also catalyzes a γ -elimination reaction in which o-succinyl-L-homoserine reacts with water to form succinate, NH₃ and α -keto-butyrate. The Km for o-succinyl-L-homoserine in this reaction is 2.8×10^{-4} M.

GAMMA ELIMINATION REACTIONS. The conversion of cystathionine to cysteine, NH₃ and α -ketobutyrate is a prototype for several enzyme-catalyzed γ -elimination reactions. Insight into the mechanism of this reaction was afforded by the discovery that N-ethylmaleimide is able to react with a transient intermediate produced in the cleavage of cystathionine to form a derivative that contains the elements of maleimide and the 4-carbon chain of the substrate; other products of the reaction are cysteine and NH₃. The structure of the maleimide derivative was shown to be:



The reaction with maleimide represents a new type of pyridoxal-P catalyzed reaction, that can be described as a γ -elimination coupled to Michael addition of a β -carbanion to an electrophilic double bond.

The use of maleimide appears to provide a new chemical approach to the enzyme-bound tautomeric intermediate thought to participate in pyridoxal phosphate catalyzed reactions.

Consequent to the widespread use of N-ethylmaleimide as a protein sulfhydryl trapping agent, it has become clear that this reagent can also react with amino groups. The nature of the latter reaction has not previously been established. In collaboration with Dr. Norman Sharpless (NIAMD) the products of reaction with a variety of amines and amino acids have been isolated, and their structures determined. In every case the reactions have been found to

involve amino group addition to the double bond of maleimide. The reaction of proline is interesting because of its rapidity at physiological pH, and because the product reverts to starting materials at pH 5, and more slowly in strong acid.

DISTRIBUTION OF TRANS-SULFURATION ENZYMES The distribution of enzymes catalyzing the various kinds of trans-sulfuration reactions has been examined in several different organisms in an effort to discover possible evolutionary relationships. The transfer of sulfur from homocysteine to make cysteine is the consequence of two coupled reactions: (1) a reaction of serine and homocysteine to form cystathionine and (2) a reaction in which cystathionine is cleaved to form cysteine, α -ketobutyrate and ammonia. Enzymes catalyzing these two reactions are present in fungi and in mammalian liver; they have not as yet been detected in bacteria. In contrast, bacteria contain two different enzymes; (1) an enzyme catalyzing the synthesis of cystathionine from succinyl-o-homoserine and cysteine, and (2) an enzyme that catalyzes cleavage of cystathionine to form homocysteine, NH₃ and pyruvate. Together, these two enzymes catalyze the transfer of sulfur from cysteine to homocysteine. Only one of these latter enzymes, the cystathionine cleavage enzyme, could be found in fungi; neither enzyme has been detected in mammalian tissues.

Mechanism of Action of Vitamin B₁₂ Coenzyme

ETHANOLAMINE DEAMINASE. An enzyme catalyzing the vitamin B₁₂ dependent deamination of ethanolamine to form acetaldehyde is the subject of a continuing investigation. The enzyme has been purified 80-fold from extracts of an ethanolamine fermenting clostridium, previously isolated in this laboratory. A highly sensitive assay procedure for this enzyme was developed in which the acetaldehyde formed from the enzymatic deamination of ethanolamine is reduced to ethanol in the presence of added alcohol dehydrogenase and DPNH. The oxidation of DPNH associated with the acetaldehyde reduction is measured spectrophotometrically. The partially purified enzyme is relatively unstable; it loses half of its activity over a

24 hour period at 0°. Partial protection against inactivity is achieved by adding ethanolamine. Preliminary studies with sucrose gradient sedimentation indicate the enzyme has a molecular weight of about 250,000. When obtained in a relatively pure form, this enzyme will be used for more detailed studies on the mechanism of action of vitamin B₁₂ coenzymes.

Anaerobic Hydroxylation of Aromatic Compounds

HYDROXYLATION OF PHENYLALANINE AND NICOTINIC ACID. The discovery that *Clostridium glycolicum* requires phenylalanine but not tyrosine for growth, and the demonstration that 6-hydroxynicotinic acid is an early intermediate in the anaerobic dissimilation of nicotinic acid by a *Clostridium* sp., suggested that these organisms might have the capacity to catalyze anaerobic hydroxylation of aromatic compounds. The hydroxylation of aromatic compounds has hitherto been regarded as an obligatory oxygen-dependent process and therefore restricted to aerobic organisms.

Encouraged by previous results showing that isotopically labeled tyrosine is present in the protein of *C. glycolicum* when grown on a medium containing uniformly labeled phenylalanine, efforts were made to demonstrate the conversion of phenylalanine to tyrosine by cell suspensions. Although cell suspensions catalyze the anaerobic decomposition of phenylalanine to a variety of substances, tyrosine could not be identified among the products. In order to obviate ambiguities inherent in the previous results obtained with uniformly labeled phenylalanine, studies are now in progress to determine if isotope from ring-labeled phenylalanine is incorporated into protein during the growth of *C. glycolicum*.

The enzyme from the *Clostridium* sp. that catalyzes the hydroxylation of nicotinic acid was partially purified and some of its properties were determined. It catalyzes a reaction between TPN and nicotinic acid to form TPNH and 6-hydroxynicotinic acid. The reaction can be followed spectrophotometrically by measuring the absorbancy at 340 m μ due to TPNH accumulation. The reaction requires the presence of ferrous sulfate and glutathione and is

markedly stimulated by the presence also of orthophosphate or inorganic arsenate. Other salts cause little or no stimulation. The stability of the enzyme at 0° and -10C is uniquely influenced by various salts. Thus at -10° stability in the presence of 0.1 M salts decreases in the following order: KHPO₄(pH 7.3) > Tris. Cl > NaCl > LiCl > KCl > NH₄Cl > imidazole Cl. However, at 0° C the order is very different; i.e., KHPO₄ > NH₄Cl > KCl > NaCl > Tris Cl > LiCl > imidazole Cl. Further purification of the enzyme is in progress to determine if it contains an unusual prosthetic group that could help to explain its unique biochemical function.

The Uptake of Amino Acid by Cellular Membrane Preparations

Isolated cytoplasmic membranes from wild type *E. coli*, strain W and from a mutant strain WS that is deficient in the ability to transport glycine, are being studied in order to determine the mechanism of carrier-mediated transport of amino acids. The membrane preparations are prepared by osmotic shock of protoplasts.

Chemical and enzymatic determinations combined with electron microscopy show that these membrane preparations have little or none of the cytoplasmic constituents of intact cells. Thus, they contain less than 1% of the total cellular DNA and RNA and only 20% of the total cellular protein. Furthermore the isolated membranes have morphological characteristics, as seen in the electron microscope, of a bimolecular lipid leaflet with a thin layer of external material.

Glycine uptake by membranes of the wild type strain is a hyperbolic function of the external glycine concentration, approaching saturation at external concentrations of 5 μ g per ml; this suggests that a carrier mediated process is involved. This conclusion is further supported by the fact that glycine uptake is dependent upon an energy source (i.e., glucose or ATP or DPNH or TPNH or succinate, etc.); it is inhibited by a variety of metabolic inhibitors some of which are known to inhibit oxidative phosphorylation; it is inhibited by alanine, serine and threonine but not by other amino acids;

alanine, serine and threonine but not other amino acids, added to the suspended medium cause rapid exchange of glycine previously taken up by the membranes; uptake is markedly influenced by temperature.

In contrast, the uptake of glycine by membrane preparations of the mutant strain WS is a linear function of the external concentration over a broad range (0–10 µg per ml), and is relatively insensitive to temperature (0–37° C); it is non-energy dependent and is not influenced by metabolic inhibitors or other amino acids.

These results indicate that membrane preparations are promising material for studies on the mechanism of carrier-mediated transport of amino acids. In future studies, efforts will be made to (1) establish the chemical nature of the intracellular exchangeable and nonexchangeable amino acid pools, (2) to determine the nature of the energy-linked process involved in transport and (3) to elucidate the character of the functional defect in the mutant strain WS.

The Metabolism of Heterocyclic Compounds

CHEMISTRY OF RIBOFLAVIN. To facilitate enzyme studies on the metabolism of heterocyclic compounds and also to explore techniques that may be applicable to projected studies on free radical intermediates in electron transport processes, investigations are being made on the chemistry of riboflavin and related isoalloxazine derivatives. Hydroxyethylflavin was obtained by periodate degradation of the ribityl group followed by borohydride reduction. When hydroxyethylflavin was treated with thionyl chloride, a facile cyclization involving the hydroxyethyl side chain and the N-atom at position 1 of the isoalloxazine ring occurred. The structure of the cyclized product was deduced from NMR, ultraviolet and visible spectral data. The compound was quite unstable in aqueous base, probably undergoing cleavage of the pyrimidine ring. Like other isoalloxazine compounds, the cyclized product could be reduced by zinc and acid to give a semiquinone as observed by ESR spectroscopy. Riboflavin also reacted with thionylchloride to give a complex mixture of products that were not fully characterized. How-

ever, 6,7-dimethyl-8-hydroxy-ethylillumazine a heterocyclic ring system resembling that of hydroxyethyl flavin but which lacks the benzoid ring characteristics, failed to undergo similar cyclization even under more drastic conditions.

Electron spin resonance (ESR) spectra of semiquinones of lumiflavin, hydroxyethylflavin and cyclized hydroxyethylflavin all showed characteristic hyperfine splittings. A tentative interpretation of the lumiflavin semiquinone spectrum was developed.

Nicotinic Acid Degradation. One of the steps in the anaerobic degradation of nicotinic acid by a *Clostridium* sp. is the conversion of 6-oxo-1,4,5,6-tetrahydronicotinic acid to α -methylene glutaric acid. To study this enzymic step in detail efforts were made to develop a practical chemical synthesis of the 6-oxo compound for use as a substrate. Three independent routes of synthesis were attempted which involved (1) the intermediary synthesis of an α -pyrone derivative through condensation of ethyl formyl acetate with ethyl acrylate; (2) The intermediary synthesis of α -cyanoglutarate by condensation of ethylcyanoacetate and ethyl acrylate; and (3) the intermediary synthesis of an imino ether by reaction of diethyl- α -cyanoglutarate with ethanol and hydrogen chloride. Only the latter method yielded the desired intermediate which was characterized by mass spectrum and its IR and NMR spectra. Treatment of the imino ether with potassium borohydride gave a neutral mixture containing two major components. One was identified as α -ethoxycarbonyl glutarate. The other has not been characterized.

Section on Comparative Biochemistry

The activities of the Section on Comparative Biochemistry are concentrated on the investigation of several basic biochemical problems related to lipid metabolism. The discovery in this laboratory of the acyl carrier protein (ACP), which appears to play a central role in lipid metabolism, has laid the foundation of many new experiments probing the interactions of lipids and proteins. Study of the mechanism of involvement of ACP in fatty acid biosynthesis and complex lipid biosynthesis has allowed the

elucidation of the enzymatic mechanism of these reactions. Knowledge of the individual reactions has led to new studies on regulatory mechanisms. Study of ACP itself has led to the elucidation of its active site, a prosthetic group, 4'-phosphopantetheine. The finding of 4'-phosphopantetheine as a component of a protein has led to a search for other proteins with similar prosthetic groups. In addition, interest has now focused on the general significance and mode of biosynthesis of such pantothenate-containing proteins.

Structure and Substrate Binding Site of ACP

All of the reactions of fatty acid synthesis in *E. coli* occur with the substrates bound in thioester linkage to the single sulphydryl group of ACP (see below). The nature of the substrate binding site was studied by labeling this site enzymatically to form C¹⁴-malonyl-S-ACP and then subjecting the protein to peptic hydrolysis. Several C¹⁴-peptides were isolated by Dowex-1 column chromatography and paper electrophoresis. Each of these peptides was shown to contain unimolar amounts of 2-C¹⁴-malonate, 2-mercaptopethylamine, β -alanine, pantoic acid, and phosphate, as well as a variety of amino acids. Demonstration of the pantoic acid required treatment of the peptides with Pronase (a proteolytic enzyme) followed by gas-liquid chromatography, whereas demonstration of the other components was accomplished by routine analyses. Although 2-mercaptopethylamine, β -alanine, pantoic acid and phosphate are important components of CoA, ACP peptides lacked pyrophosphate, ribose and adenine, indicating that the substrate binding site of ACP resembled, but was not the same as, CoA. Hydrolysis of ACP in 3N NaOH at 102° yielded 4'-phosphopantothenic acid, which, after treatment with *E. coli* alkaline phosphatase, yielded equivalent amounts of inorganic phosphate and pantothenic acid (assayed microbiologically with *Lactobacillus arabinosus*). In order to identify the entire prosthetic group intact, ACP was hydrolyzed at pH 12 (70°) for one hour, thereby liberating 4'-phosphopantetheine from the protein. The 4'-phosphopantetheine was identified by conver-

sion enzymatically to CoA with ATP, dephospho CoA pyrophosphorylase and dephospho-CoA kinase. CoA was assayed with phosphotransacetylase. These experiments established that 4'-phosphopantetheine is the prosthetic group and substrate binding site of ACP.

Further studies utilizing alkaline and enzymatic hydrolysis of this prosthetic group indicate that 4'-phosphopantetheine is bound in phosphodiester linkage to a serine residue to ACP. The following compositions have been determined for two C¹⁴-peptic peptides isolated from 2-C¹⁴-malonyl-S-ACP:

- (1) Asp, Leu, Ser, 4'-phosphopantetheine
- (2) Ala, Gly, Asp, Leu, Ser, 4'-phosphopantetheine.

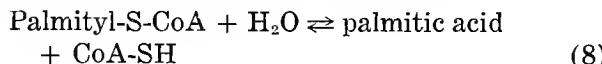
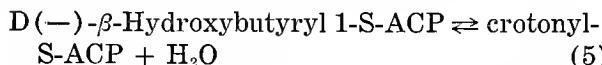
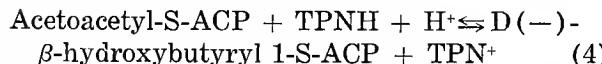
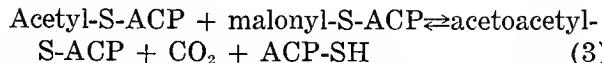
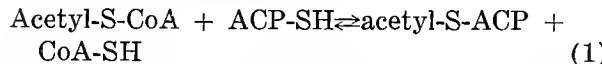
Using the Edman degradation with subtractive analysis and also fluorodinitrobenzene the sequences of the two peptides were shown to be Asp-Ser-Leu and Gly-Ala-Asp-Ser-Leu, respectively. Treatment of peptide 2 with mild alkali caused β -elimination of 4'-phosphopantetheine from the serine which was converted to dehydroalanine. Subsequent acid hydrolysis led to the formation of pyruvic acid (assayed with lactic dehydrogenase), thus establishing the linkage of 4'-phosphopantetheine to ACP as a phosphodiester linkage to the serine hydroxyl group. Additional studies of ACP have shown that the N-terminal residue is serine and the C-terminal residue is alanine.

The binding site of ACP, 4'-phosphopantetheine, is the same as that in CoA. The marked specificity of the enzymes of fatty acid synthesis for thioesters of ACP indicates that it is not the binding site of ACP but the remainder of the structure which accounts for this specificity and increased activity of ACP thioesters. Further studies of the primary and tertiary structure of ACP will be needed to delineate its mode of action.

ACP Involvement in Lipid Metabolism

ACP was initially demonstrated in extracts of *Clostridium kluyveri* and *E. coli* as a heat-stable protein required in the condensation-decarboxylation reaction of fatty acid synthesis. Subsequent studies have amplified its central

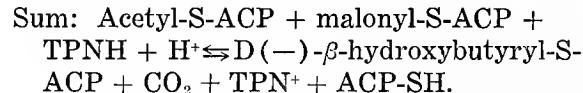
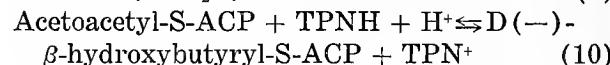
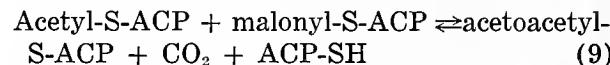
role in fatty acid biosynthesis as demonstrated by the following reactions:



As noted in the above scheme, ACP acts as the active acyl carrier in all the intermediate steps that lead to long chain fatty acid synthesis. Reactions 1 and 2 indicate the transfer of the acetyl and malonyl moieties from acetyl-CoA and malonyl-CoA to ACP to form acetyl- and malonyl-S-ACP, respectively. Acetyl- and malonyl-S-ACP condense in reaction 3 to form acetoacetyl-S-ACP, CO₂ and ACP-SH. Acetoacetyl-S-ACP undergoes reduction by TPNH to form specifically D(-)-β-hydroxybutyryl-S-ACP (reaction 4), and the latter is dehydrated to form crotonyl-S-ACP (reaction 5). Crotonyl-S-ACP is further reduced by TPNH to form butyryl-S-ACP (reaction 6). In the normal process of long chain fatty acid synthesis butyryl-S-ACP substituting for acetyl-S-ACP in reaction 3, condenses with another mole of malonyl-S-ACP thereby initiating another cycle which eventually leads to the formation of palmityl-S-ACP. Reactions 1, 2, 4 and 6 had been discussed previously. Reactions 3, 5, 7 and 8 have recently been investigated.

β-Ketoacyl-ACP Synthetase: *β*-Ketoacyl-ACP synthetase catalyzes reaction 3, the condensation of acetyl-S-ACP with malonyl-S-ACP to form acetoacetyl-S-ACP. A new assay

was developed for this enzyme based upon the following coupled system:



Coupling of *β*-ketoacyl-ACP synthetase with *β*-ketoacyl-ACP reductase (reactions 9 and 10) allows the assay of the synthetase by measuring spectrophotometrically the disappearance to TPNH in the presence of excess acetyl-S-ACP, malonyl-S-ACP, TPNH and the purified reductase. *β*-Ketoacyl-ACP synthetase was purified 192-fold by means of DEAE-cellulose chromatography, ammonium sulfate precipitation, filtration on Sephadex G-100 and chromatography on calcium hydroxyapatite. The resulting enzyme preparation, which still contained malonyl transacylase activity (reaction 2), was freed of the latter by a second filtration on Sephadex G-100. This preparation was also free of acetyl transacylase activity (reaction 1).

The synthetase has an absolute requirement for fatty acyl-ACP and malonyl-S-ACP, and it is stimulated by 2-mercaptoethanol and EDTA. Neither fatty acyl-CoA nor malonyl-CoA can replace the ACP derivatives unless the appropriate transacylase and free ACP-SH are added. Michaelis constants for acetyl- and malonyl-S-ACP are both about $2 \times 10^{-5}\text{M}$.

The synthetase was shown to be a sulphydryl protein on the basis of its requirement for 2-mercaptoethanol for maximal activity as well as inhibition of activity by low levels of sulphydryl poisons such as iodoacetamide. No inhibition was obtained with arsenite or cadmium chloride, indicating that no functional dithiol groups are present in this enzyme. The inhibition by iodoacetamide could be prevented by prior incubation of the enzyme with acetyl-ACP. Acetyl-CoA and malonyl-ACP were unable to prevent inhibition. These results indicate that acetyl-ACP is bound to the protein in such a manner as to prevent alkylation by iodoacetamide. Experiments are now being con-

ducted in order to isolate stoichiometric amounts of this enzyme so that the mechanism of this reaction can be further investigated.

ENOYL-ACP-HYDRASE. Enoyl-ACP hydrase, an enzyme which catalyzes the reversible hydration of crotonyl-S-ACP to form D(−)- β -hydroxybutyryl-S-ACP (reaction 5), has been purified approximately 20-fold from *E. coli* extracts. This enzyme is totally inactive with thioesters of pantetheine, CoA, and N-acetyl cysteamine as substrates. The enzyme is heat stable. By using D(−)- and L(+) isomers of β -hydroxybutyryl-S-ACP, it was shown that only the D(−) isomer is active with this enzyme. Since it is known that β -ketoacyl-ACP reductase forms only the D(−) isomer also, it is clear that the D(−) stereoisomer is the one which functions in fatty acid synthesis.

TERMINATION OF FATTY ACID SYNTHESIS CYCLE. Palmityl-S-ACP (or another long chain fatty acyl-ACP) is formed at the end of the sequence of reactions represented by reactions 1-6. The conversion of palmityl-S-ACP to palmitic acid could occur through direct deacylation of palmityl-S-ACP, or by an intermediate acyl transfer from ACP to CoA to form palmityl-S-CoA (reaction 7) followed by deacylation of palmityl-S-CoA (reaction 8). Evidence favors the latter pathway since enzymes which catalyze both reactions 7 and 8 have been demonstrated whereas direct deacylation of palmityl-S-ACP is not catalyzed by *E. coli* extracts.

PHOSPHATIDIC ACID SYNTHESIS. The discovery of the role of ACP as acyl carrier in fatty acid synthesis has stimulated reinvestigation of other reactions in which acyl thioesters of CoA are involved in metabolism. Important among these are the reactions which lead to the formation of phospholipids and triglycerides. Experiments were undertaken to determine whether the acyl group linked to ACP at the end of chain elongation can be transferred directly to three carbon precursors of phospholipids or if the intermediate formation of acyl-CoA is necessary. A particulate enzyme system from *E. coli* was found which catalyzes the conversion of α -glycerophosphate to phosphatidic acid in the presence of palmityl-CoA. Chemically synthesized palmityl-ACP could not re-

place the acyl-CoA derivative as acyl group donor in this reaction. Therefore it appears that the acyl group of ACP must be transferred to CoA prior to its utilization in phosphatidic acid synthesis. An interesting preliminary observation, in connection with these experiments, is that the particulate enzyme system which catalyzes phosphatidic acid synthesis contains pantothenic acid.

Involvement of ACP in the Regulation of Fatty Acid Biosynthesis

NONIDENTITY OF ACP AND LIPOGENIN. Studies on the regulation of fatty acid biosynthesis have been continued. Fatty acid biosynthesis is known to be severely depressed in liver extracts from starved rats. Dr. Herbert Anker (University of Chicago) isolated from yeast a polypeptide (lipogenin) which can stimulate fatty acid synthesis in starved rat liver preparations. Evidence suggesting the presence of lipogenin in boiled rat liver mitochondria was also reported. Anker more recently reported that yeast lipogenin has physical properties similar to *E. coli* ACP and that ACP can substitute for lipogenin in the starved rat liver assay system. Similar experiments were attempted in this laboratory. However in no instance was ACP capable of stimulating fatty acid synthesis by the starved rat liver homogenate. Since the assay system was not reproducible enough in this laboratory, a more direct approach to the problem was made. The discovery that *E. coli* ACP contains 4'-phosphopantetheine as a prosthetic group allowed a direct testing of Anker's most purified lipogenin for the presence of a similar prosthetic group. Treatment of ACP with strong alkali at 102° convert the prosthetic group to free 4'-phosphopantetheine acid which can be converted to pantothenic acid with *E. coli* alkaline phosphatase. The latter can be identified in a microbiological assay. Subjecting purified lipogenin to this treatment yielded no pantothenic acid. Therefore it is concluded that lipogenin is not identical with ACP. It can be argued that lipogenin is similar to ACP except that the prosthetic group is bound through a linkage that is not hydrolyzed by alkali, or perhaps lipogenin might represent apoACP—i.e., ACP minus the

prosthetic group, or that lipogenin does not require a prosthetic group. None of these possibilities seem probable.

FEEDBACK INHIBITION. Recent reports from a number of laboratories have indicated that fatty acid synthesis is sensitive to feedback inhibition by long chain acyl-CoA derivatives such as palmityl-CoA. One site of feedback inhibition is at the level of malonyl-CoA synthesis by acetyl-CoA carboxylase. At least one other enzyme of the six enzymes required for fatty acid synthesis from acetyl-CoA and malonyl-CoA in *E. coli* is subject to severe feedback inhibition. The enzyme, β -ketoacyl-ACP reductase, is competitively inhibited by very low concentrations of palmityl-CoA. The interaction of this enzyme and compounds which act as feedback inhibitors is under study. In connection with this work the observation has been made that many apparently unrelated enzymes are inhibited by palmityl-CoA. The physiological significance of inhibition by long chain acyl-CoA derivatives in general is being evaluated.

Biosynthesis of ACP

The demonstration that ACP contains 4'phosphopantetheine as a prosthetic group has opened the question of the biological synthesis of holoACP. It is clear that the prosthetic group is linked through phosphodiester linkage to a serine hydroxyl of the protein. In order to study the mechanism of synthesis, apoACP is required. Several approaches have been attempted to obtain this. Chemical hydrolysis of the prosthetic group from the protein has been unsuccessful because the procedure destroys the serine residue. All available known phosphatases have been tested, but none so far has been found to remove the prosthetic group from ACP. The most recent approach, and the one most likely to succeed, is production of apoACP by an *E. coli* mutant which cannot make pantothenic acid. By growing this organism on limiting pantothenate, conditions will be found wherein a large proportion of the ACP of the organism will be apoACP. Conversion of apoACP to holoACP will be attempted with crude extracts of wild type *E. coli*.

Additional Roles for Pantothenate-containing Proteins

4'-Phosphopantetheine is the prosthetic group of at least one protein, ACP, of *E. coli*. Availability of *E. coli* mutants that require pantothenic acid for growth permitted the administration of C¹⁴-pantothenate to growing cells in order to establish the fate of this compound. The cells were then opened and fractionated into a particulate fraction and a soluble fraction. The proteins of the soluble fraction were separated from CoA and other non-protein components by filtration on Sephadex. Then the proteins were further fractionated by DEAE column chromatography. At least two protein peaks contained radioactivity derived from the C¹⁴-pantothenate. In addition, the particulate fraction contained radioactivity. Experiments such as these promise to demonstrate the overall significance of protein-bound pantothenate, whether this be in the elucidation of other proteins with pantothenate prosthetic groups or in the possible implication of ACP-like proteins in such processes as cellular transport and permeation. The latter possibility has arisen with the finding that protein-bound C¹⁴-pantothenate is found in the particulate (? membrane) fraction of the cell.

Attempts to Demonstrate ACP in Mammalian Tissues

The demonstration of ACP in bacteria has stimulated a search for a similar protein in other species. Recently P. Stumpf (University of California) reported partial purification of a similar protein from plants. Both bacterial and plant enzyme preparations for fatty acid synthesis are soluble, and thus isolation of individual native protein components is relatively simple. Fatty acid synthetase preparations from yeast or mammals are protein complexes, and fractionation of these complexes to yield active enzyme components or ACP has not yet been achieved. However, the demonstration of 4'phosphopantetheine as the prosthetic group of *E. coli* ACP suggested a new approach to the question of whether the mammalian fatty acid synthetase preparation contains ACP. With the availability of a microbiological assay for pantothenate (which is derived from phosphopan-

tethine as detailed above), purification of the rat enzyme which catalyzes fatty acid synthesis could be correlated with the pantothenate content of the enzyme preparation. It was found that pantothenate copurifies with the fatty acid synthesis activity. In addition, C¹⁴-pantothenate was fed to pantothenate deficient rats, and the radioactive component was isolated from the purified enzyme system. This labeled component was shown to be 4'-phosphopantetheine by column chromatography and by enzymatic conversion to CoA. It is concluded that the mammalian fatty acid synthetase contains protein-bound 4'-phosphopantetheine. Demonstration of the entire mammalian ACP awaits further experiments.

Section on Cellular Physiology

The Cellular Physiology Section, in pursuing its program directed at the structural basis of the biochemical activities of proteins, their biosynthesis and functional relationships in the integrated activity of cellular structure, is broadly divided into three main projects:

- (1) Studies on protein structure. These are primarily focused on the fibrous proteins involved in muscular contraction and blood clotting, with major concern for substructure, and relationship of primary structure to the Configurational requirements of biochemically significant aggregation processes and enzyme activity.
- (2) Protein biosynthesis. Investigations in this area are concerned with the role of cellular membranes, the biosynthetic unit and energetics.
- (3) The metabolic relationships of unsaturated fatty acids. Investigations in this program are concerned with the metabolism, synthesis and function of unsaturated fatty acids in liver and a number of nucleated unicellular organisms.

Muscle Proteins: Myosin

Previous reports have summarized investigations leading to a model for the molecule as a long three-stranded rope with a globule at one end and composed of three physically and chemically identical polypeptide chains. Other in-

vestigations have located the enzymic sites of the molecule in the globular portion and the amino terminal ends of the chains were tentatively identified as histidine and localized in this portion of the structure as well. Previous identification of N-terminal histidine was based on an analytical procedure not entirely specific for histidine. Current activities have verified that amino terminal histidine is indeed recovered from myosin and its quantitation for the whole molecule and the globular end is in good agreement with the molecular weight of the subunit chains.

Previously, methods have been developed for selectively labelling with radioactive sulphydryl reagents each of the two cysteine residues associated with the active sites of myosin. Enzymic digestion and chromatographic fractionation and purification of the resulting peptide fragments have led to identification of a heptapeptide involving one of the two cysteines and having the sequence: glutamyl glycyl isoleucyl arginyl isoleucyl cysteinyl arginine. The second cysteine residue is present in a tryptic nonapeptide with the composition and partial sequence: cysteinyl aspartyl glutaminyl (glycyl, glycyl, valyl, isoleucyl, leucyl) arginine, the sequence of the portion in parenthesis not having been established.

Furthermore, most of the cysteine containing peptides resulting from tryptic digestion of C¹⁴-iodoacetate labelled myosin have now been isolated and at least partial sequences established for the majority of them. Successful completion of this portion of the project in the very near future should provide final proof for the chemical identity of the polypeptide chains, at present based on three less direct lines of evidence.

Bovine Fibrinogen

Earlier studies established that fibrinogen has the same molecular weight both in the presence of guanidinium chloride, which destroys the noncovalent bonding in proteins, and in dilute salt solutions. However, it is well recognized from chemical studies that fibrinogen possesses 6 polypeptide chains. Determination of the molecular weight in guanidinium chloride following cleavage of the disulfide bonds indicated that the molecule was composed of

six polypeptide chains of essentially identical mass and indicating that the six chains of fibrinogen are all associated through interrelated disulfide bridges.

Analysis of the cleavage of fibrinogen by trypsin indicated that short digestion led to formation of three fragments of equal size which appears to result from the cleavage of the three beaded structure seen in electron microscopy. Examination of the loss of clotting ability during the liberation of these three fragments indicated that loss of clotting ability occurs much faster than the cleavage reaction. However, this relationship is complicated by the fact that trypsin will split the thrombin-specific bonds in fibrinogen. Turning to chymotrypsin with its very different peptide bond specificity, this enzyme also yields the same overall picture of digestion with initial release of three large fragments. In contrast to trypsin, the loss of clotting ability parallels the release of the fragments. Following fragmentation and loss of clotting ability, it was shown that thrombin will still split four peptide bonds per original fibrinogen molecule—the same as with native fibrinogen—indicating that fragmentation has not altered the subtle specificity requirements of thrombin.

Ribonuclease

Previous work on the reaction of the fluorescent dye 5-dimethylamino-1-naphthalensulfonyl chloride (DNS chloride) with ribonuclease has demonstrated that though the dye does not inhibit the enzyme, it interferes with the reformation of the native, enzymically active structure in reduction and reoxidation of the disulfide bridges. From proteolytic digestion and isolation of the DNS peptides, it seemed probable that this effect could be related to combination of the dye with certain specific histidine and/or lysine residues. Owing to the lability of the bound dye, some ambiguities existed as to the site of attachment in these peptides. These have now been overcome and the DNS derivative of histidine 48—the major point of reaction—has been positively identified.

In other investigations involving ribonuclease, this protein has been used as a model to study the effects of gamma radiation on the structure and biological activity of proteins.

These studies necessitated the development of a new technique for studying free radical formation—in which they are trapped by reaction with tritiated hydrogen sulfide. The affected amino acid residues are then identifiable through their radioactivity. Preliminary findings indicate that with native ribonuclease, free radical formation occurs with lysine, methionine, proline and histidine residues in decreasing order. On the other hand, structurally altered, inactive ribonuclease shows an entirely different pattern of free radical formation. Studies on enzyme activity as a function of irradiation suggest that structural changes are produced that leave active but altered forms of the enzyme.

Protein Biosynthesis

Previous reports have demonstrated the high metabolic activity of the lipid soluble amino acid and peptide fractions of hen oviduct and preliminary chemical characterization indicated they were combined in the lipid structure through both amino and carboxyl ends. Though their metabolic activity made these lipopeptide materials eligible as intermediates in protein biosynthesis, no direct evidence for this existed. Approaching the problem of obtaining peptide fragments from the lipopeptides for comparison with fragments of ovalbumin obtained by the same fragmentation procedures, it was immediately evident that the lipopeptide material was rather refractory to standard hydrolysis techniques. However, recent modifications have made possible a comparison of partial hydrolysates of the two materials by the "fingerprint" technique and have indicated, within the limitations of this method, that several of the characteristic peptides of ovalbumin may be present in the lipopeptide material.

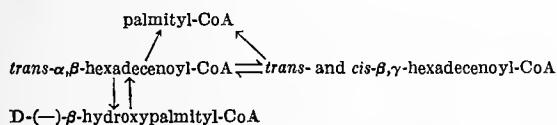
Nonphosphorylated High Energy Intermediates and Protein Biosynthesis

Recent work in a number of laboratories has indicated that the electron transport system of mitochondria gives rise to high energy intermediates which may be available to endergonic processes such as reversal of electron transport, ion accumulation and amino acid uptake prior to their involvement of phosphate and ATP formation. In the present work, pro-

tein synthesis by anaerobically growing yeast was inhibited by the classical uncouplers of oxidative phosphorylation, dinitrophenol and azide. These agents do not inhibit anaerobic glycolysis and ATP levels were the same as in the uninhibited controls. Insofar as can be detected with current methodology, there is no interference by these agents in the maintenance of amino acid and nucleotide pools. On the other hand, neither dinitrophenol nor azide inhibits the classical *in vitro* system for incorporation of amino acids into ribosomes.

Synthesis of Long-Chain Unsaturated Fatty Acids

Previous work with guinea pig liver enzyme preparations has demonstrated the formation and interconversion of long chain α,β and β,γ -unsaturated and β -hydroxy fatty acids. It has now been shown that guinea pig liver mitochondria contain two hydrases which catalyze the hydration of *trans*- α,β -hexadecenoyl-CoA to D-(—)- β -hydroxypalmitoyl-CoA. No evidence could be found for the hydration of the β,γ -CoA derivative. An enzyme preparation has been obtained that catalyzes the direct reduction of the *cis*- and *trans*- β,γ - and *trans*- α,β -hexadecenoyl-CoA compounds. All of the following reactions have now been demonstrated using partially purified enzymes.



In a study of the synthesis of polyunsaturated acids, it has been shown that a species of soil amoeba, *Acanthamoeba*, normally contains polyunsaturated fatty acids with the last double bond 6 carbon atoms removed from the methyl end of the fatty acid. When grown in the presence of linolenic acid, a C₁₈ triunsaturated acid with the last double bond 3 carbon atoms removed from the methyl end, the organism converts it to the corresponding C₂₀ tri, tetra and penta unsaturated acids suggesting that the enzyme systems for chain elongation and introduction of additional double bonds present in these organisms are sufficiently non-specific to be uninfluenced by the presence of a double

bond 3 carbon atoms closer to the methyl terminus.

In an examination of the unsaturated fatty acid metabolism of *Tetrahymena*, it has been demonstrated that this organism normally contains only 2 polyunsaturated fatty acids, linoleic and γ -linolenic acids. However, when the organisms are fed a number of unsaturated acids, many are converted to a variety of unnatural polyunsaturated acids. Some of the unnatural unsaturated acids added to the growth medium and some of the unnatural acids formed are incorporated into the phospholipids of the organism—an observation that has some implications as to the function of polyunsaturated fatty acids. It is strange that γ -linolenic acid which normally accounts for 60% of the fatty acids of the organism is lethal when added to the medium.

The Biochemistry and Cytology of Cell Transport

Previous studies on the active uptake of radioactive fatty acids by the soil amoeba, *Acanthamoeba*, demonstrated that most of the fatty acid was initially present in the cell in the form of unesterified acid, suggesting that active uptake may not involve an esterification step. The metabolic fate of the C¹⁴-palmitate has now been followed and the kinetics of its conversion to phospholipids and glycerides determined. The esterified forms appear to be largely membrane bound. The phospholipid fraction has been fractionated and is present as phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine. The fatty acid composition of each fraction has been completely analyzed.

Together with the preliminary application of electron microscopy to the cytological aspects of this process, the chemistry of some of the staining and fixation procedures commonly employed in electron microscopy have been examined. It appears that osmic acid reacts with the unsaturated fatty acyl groups to form the osmic acid esters of the dihydroxy derivatives and not with the polar portion of the phospholipids which is contrary to current concepts on which present views of the molecular orientation in cell membranes is based.

The results of studies on the stability of lipids after various fixing procedures should have wide implications in the interpretation of electron micrographs. Thus, it has been determined that after glutaraldehyde fixation, the subsequent dehydrating steps remove all of the lipids from the tissues. With $KMnO_4$ fixation, all of the neutral lipid and some of the phospholipid is removed. When OsO_4 is used, some of the neutral lipid and none of the phospholipid is removed.

LABORATORY OF CHEMICAL PHARMACOLOGY

Biological Control Systems

Adrenergic Neurochemical Transducer

In the last report a theoretical model of the control system at sympathetic nerve endings which integrates processes that synthesize, store, inactivate and release NE was described. It was termed a "neurochemical transducer" since it translates electrical impulses into a precise quantity of free neurohormone, which in turn acts on a target organ and releases mechanical or physical energy. The original structure for the adrenergic transducer, formulated by tracing out various components of the amine store with highly labeled H^3NE has stood up well. Its predictive value in expanding our knowledge of nerve function and of drug action has been great.

The following is the postulated model for the NE neurochemical transducer: NE is separated from receptors and monoamine oxidase (MAO) by a lipid membrane. The amine is present in a mobile pool which is in chemical equilibrium with a reserve pool, probably a complex of NE in granules. NE in the mobile pool is maintained within the membrane by a specialized concentrating system or NE pump. The constant concentration of NE in nerve endings results from a balance of continuous synthesis and loss of amine. MAO in mitochondria inactivates the NE that constantly diffuses from sites of storage so that the steady-state level is below that which will saturate the transport system. Nerve impulses depolarize the specialized membrane facing the receptor thereby altering the permeability to NE and releas-

ing amine to receptor sites. Between each impulse, the membrane permeability is restored and NE released onto the receptor is recaptured by the nerve ending by the action of the pump. The uptake of NE was postulated to be highly stereospecific for the 1-isomer; we have now shown this to be true. In addition, the sympathetic nerve endings retain E as well as NE but do not retain 5HT or histamine.

Release of NE by Various Drugs

In the proposed model, NE diffuses continuously from the mobile pool onto MAO at a rate proportional to concentration. Reserpine is postulated to block the NE pump, thereby increasing the rate of net efflux onto MAO. In contrast, nerve impulses and a number of drugs such as tyramine and guanethidine, are thought to depolarize the membrane directly in front of the receptor thereby releasing NE onto receptor sites. Other workers, however, hold that these drugs act on the granules themselves.

After NE synthesis is blocked by α -methyltyrosine, the concentration of amine in brain and heart declines exponentially. These results demonstrate that the passage of catecholamine from the complex in granules to the mobile pool can be explained by simple mass action kinetics.

Reserpine depletes NE by increasing its rate of efflux in the mobile pool; the loss of NE is still proportional to the concentration but the rate constant is increased. From the kinetics of NE loss, the degree to which reserpine inhibits the NE pump may be calculated.

Reserpine appears to act by removing a resistance (the NE pump) to the free flow of the monoamine from nerve endings. Accordingly by relating the NE efflux in control rats to the NE efflux when the amine is released at a maximal rate, the activity of the NE pump can be calculated. The results are consistent with the view that reserpine acts on the membrane transport rather than on the granule complex.

Tyramine, infused so as to produce a high plasma level, also depletes heart NE by increasing its efflux from the mobile pool. The loss of NE, which is released onto receptor sites as the free base, is exponential with no

evidence of a tyramine-resistant pool or of tachyphylaxis. These results indicate that the dissociation of the NE complex to readily available amine is not the rate-limiting step. The maximal rate of release ($t_{1/2} = 50$ minutes) is about one-third that after reserpine, suggesting that the NE pump has not been blocked but that the amine is released only from the membrane opposite the receptor, whereas after reserpine it is released over a larger membrane surface. Metaraminol and octopamine, *in vivo*, and guanethidine *in vitro* also release NE onto receptors as the base at the same maximal rate as tyramine, indicating that these drugs all release the amine by a similar mechanism.

The effects of MAO inhibition support the view that tyramine does not act directly on granules. Thus blockade of MAO slows the rate at which NE is released by reserpine, but does not affect that produced by tyramine.

After depletion of heart NE by tyramine, the stores of NE can be replenished by the injection of exogenous NE, showing that tyramine, and presumably guanethidine and aramine, do not affect the intrinsic storage mechanism.

Mechanisms of NE Release at Molecular and Subcellular Levels

The distribution of metaraminol *in vivo* is indicative that the drug is an almost ideal tool for studies of the mechanism that stores and releases NE. It is taken up only by adrenergic neurons and it is not bound to non-specific sites. Preliminary results suggest that the drug is distributed in almost exact proportion to the concentration of endogenous NE in tissues; the use of the labeled drug might therefore provide a simple way to compare NE levels in various tissues. Preliminary results indicate that metaraminol is readily taken up by nerve endings of rats given reserpine. However the amine disappears at a relatively rapid rate since the system is now open-ended.

Metaraminol was found to be localized in heart granules of rats pretreated with desmethylimipramine. Since this drug blocks the release of NE elicited by metaraminol, it is unlikely that the latter drug releases NE by a direct action on granules.

Heart slices, incubated *in vitro*, accumulate metaraminol by a specialized transport system. In contrast, the drug is taken up by skeletal muscle by passive diffusion. Since the steady-state level achieved by both processes is reached at the same time, the storage process may be regarded as a pump and leak process. The uptake process is saturated when the concentration of metaraminol inside heart tissue reaches 0.7 microgm/g. This level compares with the 1.1 microgm/g of NE normally present. The accumulation of drug and the release of NE appear to be separate processes; the uptake of metaraminol is relatively rapid while the release of NE is much slower. The extent of NE depletion is related not only to the level of metaraminol but also depends on the incubation time.

Heart slices from rats treated with reserpine take up metaraminol at the same rate as would be predicted from the rate constant for passive diffusion but the diffusion process continues over a long period of time until the final concentration of drug in tissue becomes equal to that in control slices. Skeletal muscle slices do not exhibit similar behavior. According to our model, these results would be explained by a slow passive diffusion of the drug together with its reaction with granules to form a complex of high affinity. Substantiation of this view would be definite proof that reserpine does not act directly on granules.

To achieve similar conditions with NE, heart slices taken from animals treated with reserpine and a MAO inhibitor (to protect the NE) are incubated with H^3 NE. The uptake of H^3 NE at equilibrium is 2.5 times that in tissues of animals that received only reserpine. These results indicate that reserpine blocks active transport at the neuronal membrane but does not act directly on granules.

The Effects of Desmethylimipramine (DMI) on Uptake and Release

DMI is proving to be a pharmacological tool of considerable importance in unravelling the nature of the adrenergic neuron. DMI, which by itself exerts no pharmacological effects, reverses reserpine depression to a pronounced excitation. This prompted us to study the effects

of DMI on the uptake and release of NE at peripheral adrenergic neurons. At first these effects seemed paradoxical, since DMI blocks the uptake of H^3NE given in tracer doses without releasing endogenous NE and without apparently blocking the uptake of large doses of NE. DMI exerts similar effects on drugs which are stored by adrenergic neurons; for example it prevents the uptake of guanethidine, metaraminol and tyramine given in small doses, but does not release these drugs after they have been taken up by nerve endings. Moreover, DMI does not block the uptake of these drugs when they have been given in large doses, but invariably prevents them from releasing NE.

The finding that a given dose of DMI blocks the uptake of H^3NE by the same proportion over a considerable dosage range suggested that DMI might decrease the intrinsic permeability of the neuronal membrane to NE. Thus, 20 mg/kg DMI blocks H^3NE uptake by 95% and would be said to block almost completely the uptake of tracer doses of the catecholamine. But the inability of DMI to block the uptake of large doses of NE and of various drugs stored by the adrenergic neuron is only apparent. The small proportion of NE or drug uptake is now sufficient to saturate the binding capacity of the adrenergic neuron.

Although DMI does not prevent the uptake of NE after large doses of the amine, it prevents this NE from displacing H^3NE already in the nerve endings. Thus DMI inhibits the passage of NE out of, as well as into, the nerve endings. Moreover it seems to separate the granule from the mobile pool. In support of the view that DMI affects membrane both at the neuronal membrane and at granules, the drug decreases the spontaneous loss (turnover) of H^3NE from rat heart. In addition it decreases the quantity of NE released by nerve impulses though it also potentiates the biological response.

The Recapture Process

The inability of nerve stimulation to deplete NE stores, despite a slow turnover of the amine, may be explained by the model. Between successive nerve impulses, the impermea-

bility of the neuronal membrane is re-established and amine released to receptors will be pumped back into nerve endings and used again. Since phenoxybenzamine prevents this re-uptake, this drug provides a good tool for a study of the recapture mechanism. In the cat-colon preparation, this drug increases about 5-fold the H^3NE output produced by electrical stimulation. It is generally believed that phenoxybenzamine blocks directly the NE uptake by nerve endings. Our results show that phenoxybenzamine, after it has acted irreversibly, blocks the uptake of H^3NE to only a minor degree. The drug prevents the attachment of NE to the receptor site and allows it to escape into the blood stream while the nerve terminal is still depolarized. In contrast, cocaine (and DMI) actually blocks the uptake of NE and in fact lowers the output of NE by nerve stimulation. Further evidence that the two drugs act differently is the finding that phenoxybenzamine increases the spontaneous outflow of H^3NE (normal tone) while cocaine diminishes it. Thus phenoxybenzamine acts by blockade of receptors, while cocaine and DMI act by blockade of the uptake. This particular action of adrenergic blocking agents should be important in differentiating alpha and beta blocking agents in biochemical terms.

The difference between the output of H^3NE after sympathetic stimulation of control and phenoxybenzamine-treated colon, has permitted the calculation of NE released per nerve impulse and of the extent to which the recaptured mechanism preserves the amine supply. Such calculations show that nerve endings would soon be depleted of their neurohormone if they lacked the reuptake process.

Rates of NE Synthesis

The rate of formation of NE may be calculated from rate of NE los after blockade of synthesis by α -methyltyrosine

$$[NE] = [NE_0]e^{-kt}$$

where NE_0 is the initial level of NE

$$\ln \frac{[NE]}{[NE_0]} = -kt$$

A plot of $\ln \frac{[NE]}{[NE_0]}$ vs time yields the fractional turnover rate constant and $k [NE_0] = \text{synthesis rate}$.

Synthesis rate in heart = 0.05 microgm/g/hr
Synthesis rate in brain = 0.12 microgm/g/hr

The rate of formation of NE in heart may also be calculated from the rate of refilling of the NE stores after depletion by tyramine.

$$[NE] = [NE_0] (1 - e^{-kt})$$

where NE_0 is the final level of NE

$$\ln \frac{[NE]}{[NE_0]} = kt$$

A plot of $\ln \frac{[NE]}{[NE_0]}$ vs time yields fractional turnover rate constant and

$$k [NE_0] = \text{synthesis rate} \\ = 0.05 \text{ microgm/g/hr}$$

Role of Inorganic Ions in Storage and Release of NE

Because of the importance of electrolytes in depolarization phenomena and in biological processes, we are now studying the effects of electrolyte changes on the storage and release of NE in heart slices taken from rats injected with H^3NE . The rapid efflux of radioactivity that occurs when the slices are first incubated with Krebs-Ringer solution, appears to result from depolarization and soon subsides. The rate of efflux of H^3NE now becomes slow and declines exponentially.

Depolarization elicited by increasing external K^+ enhances the rate of H^3NE release; at maximum depolarization, the rate constant of release is identical to that elicited by guanethidine, suggesting that the drug also releases NE membrane depolarization. Addition of bretylium to the incubation fluid blocks the release of NE elicited by guanethidine.

In the absence of external Ca, the efflux of NE depends on the ratio of K^+ to Na^+ and maximum retention of the amine is obtained in a ratio of about 10 to 1. This suggests that an

ATPase is involved in maintaining the NE pump.

The most rapid efflux of NE occurs in the complete absence of Na^+ , suggesting that the uptake process might be linked to the Na^+ pump. It is possible that the rate of efflux in this case is the same rate as that produced by reserpine.

Ouabain blocks the uptake of NE by heart slices; in addition it releases NE from heart slices in a Ca free medium.

Other Research Items

RELEASE OF DRUGS BY NERVE STIMULATION. We have continued our studies of drugs, such as tyramine and guanethidine, which are taken up by adrenergic neurons and then appear to elicit persistent depolarization. In support of the view that they are stored in sympathetic nerve endings, our results show that they are released by electrical stimulation. In addition, reserpine interferes with their uptake.

THE SUBCELLULAR ACTION OF DRUGS. The presence of NE in granules (vesicles) and the selective action of various drugs makes it possible to study drug action at a subcellular level. For example, bretylium, DMI and certain MAO inhibitors prevent guanethidine, tyramine or metaraminol in pharmacological doses from releasing NE but do not block the uptake of the latter drugs by adrenergic neurons. It may be presumed that the release of NE is prevented by an action within the neuron itself.

Bretylium or DMI might conceivably block depolarization elicited by guanethidine or metaraminol, or prevent these substances from displacing NE from granules. Since neither DMI nor bretylium prevents the uptake of metaraminol by granules, it is unlikely NE is released by metaraminol in this way.

However, the techniques for isolating granules have given such inconsistent results that this approach has been suspended in order to examine the effects of tissue fractionation on granule NE. Preliminary results suggest that the leakage of NE from synaptic vesicles, rather than breakage of vesicles, might be the main reason for the loss of NE. Steps are underway to remedy this loss.

USE OF DRUGS IN MEASUREMENTS OF NE COMPARTMENTS. Results from this laboratory have shown that the amine compartments can be traced out by means of H³NE administered in tracer doses. Conclusions about amine compartments are difficult to make if nonsteady conditions prevail. Thus, based on release of NE by reserpine and tyramine in large doses, it might be concluded that NE is present in a single compartment, since both pools are in dynamic equilibrium. Again the ratio of the efflux of H³NE relative to the efflux of endogenous NE will not disclose information on pool size but will simply show conditions under which passage from one pool to another, or the effect of the disappearance of drug itself, becomes rate limiting.

BRETYLIUM. Although bretylium, like guanethidine, prevents the nerve impulse from releasing NE, it apparently acts by a different mechanism as described in previous reports. Our studies now show that reserpine releases guanethidine but not bretylium; catecholamines can compete for the uptake of H³-guanethidine but not for that of C¹⁴-bretylium; pretreatment with large doses of guanethidine dilutes out the uptake of H³-guanethidine, but large doses of bretylium do not dilute out the uptake of H³-bretylium. Thus the two drugs are incorporated by different neuronal sites.

AN INDIRECT EFFECT OF RESERPINE ON GRANULES. After depletion of heart NE by the injection of reserpine, amine levels are completely depleted for 2 to 3 days. In about 20 hours, however, the nerve endings recover in part the ability to take up H³NE but not to store the amine. These results indicate that the NE pump recovers before the granules are able to form a stable complex with the amine, and suggest that one of the complexing components has disappeared.

The Serotonergic Transducer

A definite role has not yet been assigned to 5HT in brain because of the inaccessibility of brain neurons and the difficulty of interpreting the effects of endogenous 5HT, e.g., small doses of 5HTP elicit sedation while large doses elicit excitation. Although 5HT and NE transducers

can be described in almost identical terms, few drugs seem to have a specific action on the 5HT transducer. Thus no substance has been found that mimics, blocks the action of, or blocks the synthesis of 5HT. In fact the main clue for the action of 5HT has been reserpine, which blocks the storage of both 5HT and the catecholamines. If it could be established that sedation were attributable mainly to changes in 5HT, reserpine would assume an important role in studies of the physiological function of this amine.

The catecholamine amines are now definitely eliminated from the picture of reserpine sedation, since α -methyltyrosine, which specifically blocks the formation of catecholamines, elicits no sedation in doses that deplete the amines by 80%, and produces typical chlorpromazine-like effects when depletion is 90% or more. Last year we reported that recovery from reserpine was closely related with the recovery of brain tissue's capacity to take up 5HT formed from exogenous 5HTP. That reserpine acts through constant occupancy of receptors by 5HT is now virtually proved. First, excitation elicited by large doses of 5HTP is not due to the action of 5HT at serotonergic receptor sites but to displacement of brain NE by 5HT formed in adrenergic neurons. Second, a high concentration of free 5HT (62 ng/g) exists at nerve endings after the complete depletion of amine stores. Most importantly, after blockade of catecholamine amine synthesis, a MAO inhibitor given to rats treated with threshold doses of reserpine, elicits a profound sedation that is correlated with the rise in brain 5HT.

Reserpine no longer produces tremors in animals when catecholamines synthesis has been blocked. These results suggest that the tremors arise from persistent occupancy of dopamine receptors in caudate nucleus. This would explain why reserpine tremors are blocked by chlorpromazine but not by atropine.

Effects of Drugs in Various Phyla

The brain of the lamprey, the most primitive of fish, contains 5HT and dopamine, but no NE or E. Only in higher classes of fish does NE appear in brain. Amphibian brain contains E, but no NE. The E, however, is resistant to re-

lease by reserpine or any other drug we have tried.

The action of reserpine in lower animals is dependent upon the rate of 5HT formation. In mammals and birds for example, reserpine always produces sedation and 5HT is formed at a high rate. In amphibia, the formation of 5HT is slow; although reserpine releases 5HT stores, it does not elicit sedation unless the animals are also given a MAO inhibitor to protect the liberated amine.

In frogs, the administration of a MAO inhibitor produces sedation after a period of 24 hours or longer. At this time there is no rise in brain E, but 5HT levels have risen and have reached a peak. The following drugs elicit sedation in frogs pretreated with MAO inhibitors: α -methyl-metatyrosine, dopamine, 5HTP, amphetamine. None of these drugs elicits sedation by itself and in each case the sedation is related to the presence of a pool of free 5HT.

Nonmast Cell Histamine

Last year we reported that highly labeled H³-histamine injected in tracer amounts selectively labeled the nonmast cell histamine stores. Such histamine was found to be present in all tissues and is not released by compound 48/80. The assay of H³-histamine by isotopic dilution now makes it evident that the reported diphasic disappearance of the labeled histamine is true only in part. The second component (half-life of 150 hours) is not that of histamine but that of a metabolite of unknown structure, which is distributed evenly in body water and is poorly excreted. The kinetics of distribution of H³-histamine indicates that nonmast cell histamine has a rapid turnover, about 2 hours.

Contrary to the general impression, some animal species (cat and rabbit) possess relatively little mast cell histamine and large amounts of nonmast cell amine. For example, except for the skin, the tissues in cats and rabbits contain mainly nonmast cell histamine.

The following results, obtained mainly by experiments on the submaxillary gland, suggest that the action of the parasympathetic nervous system on exocrine glands may be mediated by nonmast cell histamine:

H³-histamine in cat submaxillary gland de-

cines with a half-life of 2 hours. Stimulation of the chorda tympani for 3 hours releases considerable amounts of labeled histamine into saliva as histamine metabolites; the H³-histamine concentration in the gland is reduced by about 80% relative to the unstimulated gland. Changes in the rate of salivation, produced by changes in frequency or voltage of electrical stimulation, are closely correlated with changes in the amount of label in the saliva.

This relationship was further established by studies showing that salivation and the release of labeled histamine are blocked by atropine, while they are enhanced by eserine. Other agents that enhance both phenomena are acetylcholine, tremorine, reserpine and pilocarpine.

Pilocarpine appears to have more than one action. It acts like acetylcholine and in addition it depletes nonmast cell histamine, at least in the submaxillary gland. Thus, pilocarpine is a valuable tool in studying histamine compartments and in relating cholinergic effects to histamine content. For example, shortly after H³-histamine is given, pilocarpine releases a large proportion of H³-histamine and only a small proportion of the endogenous amine, suggesting that histamine is located in more than a single compartment. However, after 1 to 2 hours, the labeled and endogenous amines are released to the same extent. Large doses of pilocarpine release a large fraction of endogenous amine after which the gland is refractory to stimulation of the chorda tympani. Pilocarpine also increases the flow of saliva, gastric juice, bile, pancreatic juice, tears, nasal secretion and sweat. Bile and pancreatic juice, which have been studied in detail, contain considerable amounts of labeled histamine metabo-

lites.

Infusion of the histamine analogue, Histalog, considered to be specific for secretion of gastric juice, also enhances secretion of all the exocrine glands. Priscoline produces similar responses and, in addition, elicits peripheral vasodilation. This is of particular interest in view of our ignorance of the control of microcirculation.

The polypeptide gastrin releases considerable amounts of both labeled and endogenous histamine from the stomach, but not from

other organs. This action is not blocked by atropine and suggests that gastric action may be mediated through histamine release. Secretin produces a profuse flow of pancreatic juice and bile which contain considerable amounts of labeled metabolites.

Nonmast cell histamine accounts for the bulk of the amine formed in the body. It is not clear how the processes of synthesis, binding, release and metabolism of nonmast cell histamine are integrated, but the following picture is emerging: at the steady state, nonmast cell histamine is formed and inactivated at a rapid rate, the metabolites diffusing into the blood stream. The synthesis and utilization of histamine are closely linked processes. Atropine slows down the normal turnover of labeled histamine, in addition it reduces the incorporation of H^3 -histamine from the H^3 -histidine into endogenous stores in the gastric mucosa and salivary gland. These results suggest a feedback mechanism by which histamine inhibits histidine decarboxylase, thereby permitting a precise adjustment of synthesis according to need. This view would explain why histamine stores are not depleted even after prolonged nerve stimulation. In support of this view, the turnover of histamine in the gastric mucosa is reduced to about 20% of normal, when the rat esophagus is tied off to prevent the entrance into the stomach of a salivary factor known to promote gastric secretion.

Since H^3 -histamine is released into the exocrine secretions as metabolites, histamine must be stored in the secreting cell. Moreover, acetylcholine from cholinergic nerve endings must also act on, and the metabolizing enzyme must occupy this cell. Since the usual inhibitors do not protect the histamine released by nerve stimulation, the enzyme inside the target cell is not one of those that inactivate circulating histamine. Gastrin and other polypeptide hormones of the GI tract release histamine by a different and more specific mechanism not inhibited by atropine.

Sympathetic Target Sites

Mammals are able to make constant adjustments to the environment by a unique kind of adaptation in which enzymes are activated al-

most instantaneously by the nervous system. We are now studying three tissue systems which respond to sympathetic stimulation through enzyme activation and increased mobilization of energy substrates—adipose tissue, the liver and skeletal muscle. The cells of these organs are transducer systems in which the input is NE and the output FFA, glucose or glucose-phosphate. We are particularly concerned with the way in which a physiological signal—a catecholamine released to the target cell—is converted to a biochemical signal, e.g., cyclic AMP inside the cell.

Earlier studies showed that the functionally sympathectomized rat (adrenal demedullation and loss of peripheral stores) is unable to mobilize energy substrates. Such animals rapidly die on exposure to cold and collapse after forced exercise. The fact that these animals respond like adrenalectomized (ADX) rats led to studies which demonstrated that the sympathetic nervous system of these animals was unable to respond to catecholamines.

Adipose Tissue System

Since catecholamines, ACTH, and glucagon can elicit the same maximum rate of lipolysis, it is generally assumed that the rate of this process is limited by the amount of lipase. The finding that this rate is increased by theophylline led us to study this drug in some detail.

Incubation of theophylline with adipose tissue or with fat cells produces a lipolytic response (as measured by glycerol output) having a maximum 3 times that yielded with NE or E. Theophylline, in an amount that elicits no response by itself, potentiates the lipolysis induced by NE and ACTH, but the resulting activity never exceeds the maximal response elicited by theophylline itself. These results suggest that the activation of lipase by catecholamines is normally limited by the low steady-state level of cyclic AMP and that theophylline, by blocking phosphodiesterase, causes cyclic AMP to accumulate to a concentration that produces a maximal and perhaps a complete activation of lipase. In direct support of this view, theophylline causes a remarkable accumulation of cyclic AMP, which is closely associated with the lipolytic response; at a high concentration

of theophylline, the cyclic AMP level is increased by 6 times. Of particular interest is the finding that the maximum effect of theophylline is diminished in high doses; a similar finding has been reported for the action of cyclic AMP on lipase. Since theophylline potentiates NE and ACTH to the same degree, ACTH as well as NE may act through cyclic AMP.

Interaction of Sympathetic and Thyroid Systems

This action of theophylline makes it a valuable tool in determining whether a physiological change in lipolytic activity is caused by a change in lipase or in the ability to activate the enzyme. The effects of theophylline on the adipose tissue of thyroidectomized rats indicate that the slow rate at which cyclic AMP is formed is responsible for the poor responsiveness to catecholamines. In addition, the hyperresponsiveness of adipose tissue from hyperthyroid rats results from the rapid rate at which cyclic AMP is formed. These results suggest that the interaction of the thyroid and sympathetic systems might involve induction of adenyl cyclase. In accord with this view, the activity of adenyl cyclase in rats is tripled after pretreatment of rats with thyroxine, and this activity is closely related to the lipolytic response to NE.

Nicotinic Acid-Induced Inhibition of Lipolysis

In vitro studies show that nicotinic acid, unlike the adrenergic blockers, inhibits the response to theophylline much more readily than to NE. Inhibition occurs at concentrations of 10^{-7} M and maximum inhibition at about 10^{-5} M; in higher concentrations the effects are diminished. The addition of theophylline reduces the action of nicotinic acid indicating competition between the two drugs. These results suggest that nicotinic acid might activate phosphodiesterase. This view is supported by results which show that when nicotinic acid is added to an incubation medium containing phosphodiesterase and cyclic AMP, the hydrolysis of the latter is increased.

Possible Mechanisms of Alpha and Beta Adrenergic Blockade

The lipolytic action of low concentrations of dichloroisoproteranol (DCI) is barely measur-

able when the latter is used alone, but is markedly potentiated by low concentrations of theophylline.

In higher concentrations, both DCI and phentolamine inhibit the lipolytic effects of NE and theophylline. DCI acts competitively against NE and is much more effective in blocking the response to NE than that to theophylline. In contrast, phentolamine blocks the NE and theophylline effects to the same extent and acts noncompetitively against NE. These results suggest that DCI acts primarily at the receptor site and that phentolamine acts primarily at a step between the production of cyclic AMP and the activation of lipase—perhaps on the action of cyclic AMP itself. In very large doses, DCI also blocks the effects of theophylline but this effect may be secondary.

Lipolytic Effects on Theophylline in Vivo

Theophylline *in vivo* produces a marked increase in the plasma levels of both FFA and glucose, and together with NE elicits a more than additive increase in FFA. The maximum effect of theophylline alone or theophylline together with NE is greater than the maximum response to NE alone.

Of great potential importance is the finding that the maximal effects of theophylline in chemically sympathectomized rats, or on fat pads from these animals, are practically the same as in intact animals. This indicates that the sympathetic nervous system is not required for the action of theophylline. It may be concluded that cyclic AMP is continuously released irrespective of the nervous system. It is possible that changes in this basal turnover might explain the changes in lipolytic activity that occur in metabolic states like starvation.

Biochemical Lesions in Adrenalectomy

Epinephrine and glucagon increase the plasma glucose level in normal but not in ADX animals. ADX animals maintained for a couple of days on normal saline respond to epinephrine and glucagon with a moderate increase in blood glucose, but 3 hours after pretreatment with a glucocorticoid, respond normally. Liver phosphorylase activity also increases after epinephrine or glucagon treatment in normal

animals but in ADX animals, maintained on water, the increase in phosphorylase is far less significant. Pretreatment with a glucocorticoid restores the response to that in normal animals.

Cyclic AMP level in liver is increased about 2-fold one minute after epinephrine or glucagon injection in normal animals but is not increased in ADX animals. After pretreatment with corticoids the response is partly restored.

Cyclic AMP does not penetrate cells in appreciable amounts except after large doses. Five minutes after injection of 10 mg/kg of cyclic AMP, the concentration of label in liver is about 5 times that in muscle and 20 times that in blood and a moderate increase in blood glucose is elicited. The effect is less dramatic and quite variable with ADX animals. Cyclic AMP activates liver phosphorylase to the same extent in normal and in ADX animals maintained on water.

These results suggest that the biochemical lesion in ADX animals is centered around cyclic AMP. The reactivity of animals to epinephrine might depend on an optimum ionic environment which is lacking in ADX animals and is restored by corticoids.

In normal and ADX animals, theophylline but not NE is able to mobilize glucose and FFA. Preliminary studies indicate that theophylline increases the cyclic AMP content of liver about 6-fold. This suggests that the action of theophylline in mobilizing glucose is mediated through cyclic AMP. The level of cyclic AMP in adipose tissue is also increased to some extent.

Factors Which Affect Drug Action

Embryotoxic Effects of Thalidomide

The mechanism by which thalidomide exerts toxic effects on the embryo is still obscure. It is known that the drug is hydrolyzed nonenzymatically in the fetus to polar metabolites and it has been assumed, but not proved, that these metabolites are the cause of the embryotoxic effects of thalidomide.

Our results show that labeled thalidomide, injected intravenously into rabbits (5 mg/kg), disappears with a half-life of about 2 hours.

The lipid-soluble drug readily crosses the placenta at a rate that is presumably related to the plasma level. Accordingly, most of the drug that enters the fetus does so shortly after its injection and is hydrolyzed nonenzymatically to a variety of metabolites, which are highly polar and are trapped within the placental barrier. In 4 hours the concentration of total metabolites in the rabbit fetus reaches about 100 $\mu\text{g/g}$, which is 500 times greater than that of unchanged thalidomide in fetal tissue, or plasma of mother (0.18 $\mu\text{g/ml}$). By giving the thalidomide intravenously, reproducible teratogenic effects are obtained in rabbits with doses ranging from 2.5 to 10 mg/kg (daily from the 6th to the 10th day of pregnancy). In contrast, data from the literature show that 4 hours after oral administration (100 mg/kg), the plasma level of the drug is about 4 $\mu\text{g/ml}$ and the total metabolites in fetus reach values equivalent to about 7 $\mu\text{g/g}$. Since large doses given orally are necessary to cause teratogenic effects in rabbits, the embryotoxicity is apparently more closely related to the fetal level of metabolites than to the level of the parent drug.

These results indicate the importance of designing experiments with thalidomide that take advantage of the lipid solubility of the parent drug and the polar nature of possibly toxic metabolites. Since the metabolites in the fetus must originate from the parent drug, it is important that the parent drug attain a high plasma level in the mother. This is best done by intravenous injection.

The literature shows that huge oral doses of thalidomide are needed in the rat to attain a teratogenic effect. Our results show that the drug has a potent teratogenic effect in rats after intravenous injection.

Since the drug disappears in rats and rabbits at similar rates, these results suggest that the teratogenic effects in rats depend on the plasma levels shortly after i.v. injection. It is commonly believed that thalidomide and its metabolites cause malformations by interfering with glutamic acid metabolism, but this may now be questioned since phthalimido-phthalimide, which does not contain a glutarimide ring, is more embryotoxic than thalidomide in rabbits.

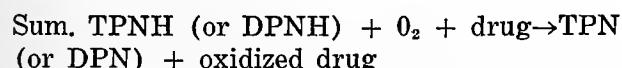
Desmethylimipramine

The cholinergic effects of tremorine are mediated through the formation of oxotremorine and not by the parent drug. Last year we reported that desmethylimipramine (DMI) delays the onset of the tremors induced by tremorine, but prolongs the action of oxotremorine. We have now definitely established that DMI in rats affects the oxotremorine-induced syndrome by inhibiting the metabolism of oxotremorine to inactive metabolites; pretreatment of rats with DMI impairs the metabolism of oxotremorine by liver preparations.

Enzymatic Mechanism of Drug Metabolism

Evidence obtained in this and other laboratories indicates that the component in liver microsomes, which reacts with O_2 to form an "active oxygen" complex, involves a hemin pigment which can be identified by its ability to combine with CO to form a complex having an absorption maximum at 450 m μ . This pigment is called P-450. Carbon monoxide inhibits the oxidation of drugs by microsomes, as well as the oxidation of TPNH in the absence of drugs. In addition, ethylisocyanide which combines with P-450 is a good inhibitor. There is also a good correlation between binding of P-450 and inhibition of drug metabolism. Thus, the oxidation of drugs may be as follows.

1. TPNH + oxidized P-450
TPN-cytochrome c reductase →
TPN + reduced P-450
- 1.a DPNH + oxidized P-450
DPN-cytochrome c reductase →
DPN + reduced P-450
2. Reduced P-450 + $O_2 \rightleftharpoons O_2 - P-450$
3. $O_2 - P-450 + \text{Drug} \rightarrow \text{oxidized P-450} + \text{oxidized drug}$



P-450 is reduced more rapidly by TPNH than by DPNH, which accounts for the apparent specificity of the enzymes for TPNH. In addition, P-450 is involved in the anaerobic reduction of nitro- and azo-compounds, since CO

blocks the reduction of p-nitrobenzoic acid and prontosil.

Compounds that block the drug-metabolizing enzymes may interfere with P-450 in various ways. For example, incubation of mouse liver microsomes with a TPNH generating system and either SKF-525A or Lilly 18947 causes the formation of a substance, which has an absorption maximum at 450 m μ , and is apparently an inactive form of P-450. JB 516 does not give rise to the 450 m μ -absorbing substances, but apparently destroys microsomal P-450 even without preincubation with microsomes. Finally, DPEA, the primary amine analogue of Lilly 18947, apparently acts by inhibiting the reduction of P-450.

Passage of Substances Across Membranes

Central Nervous System

An important problem concerns the mechanism by which the CNS disposes of polar metabolites for the failure of such processes might lead to abnormal brain function. Our studies indicate that there is a saturable, active process in rabbits that rapidly transports choline and other quaternary amines. The site of this transport is the choroid plexus which *in vitro* accumulates the amine by a process having all the characteristics of active transport. 5HT, NE, hexamethonium, decamethonium, and NMN share this process, suggesting that the choroid plexus "pump" is important in disposing of organic bases from the CNS.

Further studies have been carried out with the active transport system in brain which transfers 5-hydroxy-indolacetic acid (5HIAA) directly from brain to blood. This process may be blocked by probenecid. The block is complete since the accumulation of 5HIAA in brain over a period of two hours is consistent with the known rate of formation.

Preliminary results indicate that the ependymal membrane is highly permeable to choline, as well as hexamethonium, inulin, and mannitol which pass from CSF to brain and presumably from brain to CSF by simple diffusion. This process is far slower than transport from CSF to blood.

Biliary Excretion of Drugs

The liver is known to transport organic acids and organic bases into bile by different systems. A third transport process has been discovered that secretes ouabain, a nonionized compound, at a rapid rate ($t_{1/2}$ 13 minutes). Dihydro-ouabain is also excreted like ouabain, but at a slower rate. This process is not inhibited by organic acids or bases. Preliminary results indicate that liver slices accumulate ouabain by an active transport process.

Chlorothiazide, an organic acid lacking carboxyl or sulfonyl groups, shares the same secretory process as other acids. Thus, the carboxyl or sulfonyl acid groupings are not required for transport.

Enzymatic Mechanism of Membrane Transport and Mechanism of Hormonal Control of Active Transport

Since physiological control of the cardiovascular system involves operations on the cell membrane, the general theme will be to provide molecular descriptions of 1) the mechanism for maintaining excitability of nerve and muscle by the ion transport systems in the membrane and 2) the molecular changes in susceptible membranes caused by the impact of neurohumoral transmitters and various drugs.

Since there is abundant evidence that a membrane ATPase in nerve and muscle provides the enzymatic machinery for ion transport, most current efforts are devoted to a study of the mechanism of this reaction within the limitations imposed by the impurity of particulate preparations now available. An effort is being made to isolate proteins of sufficient purity for physical chemical studies of the structural changes which are presumably associated with the ion carrier function. With the exception of relaxation methods, which are not now being used in the NIH, the physical chemical investigations of proteins will be carried out in collaboration with other groups.

The identification of the phosphorylated intermediate whose formation is catalyzed by sodium, as a mixed anhydride of phosphoric acid and an acidic group of the protein, will permit rapid progress in studies of the active center of transport ATPase. The fact that this is a high

energy intermediate suggests that the actual conversion of phosphate bond energy into osmotic work must be associated with the potassium-catalyzed dephosphorylation of the enzyme. The high reactivity of the intermediate with amines and hydroxylamines should permit its ready conversion to a stable isotopically labeled derivative that can be used to identify the particular amino acid that serves as the phosphate carrier. This work is now continuing.

The second potassium-catalyzed part of the reaction may be studied independently of the sodium-sensitive intermediate formation by means of artificial substrates. This now makes it possible to determine by fractionation studies whether the transport ATPase system is a single enzyme or a family of enzymes associated on a matrix in the membrane. If the latter is the case, the use of artificial substrates may be the key to the isolation of pure components of the system.

Stimulation of the synthesis of transport ATPase in the kidney of the adrenalectomized rat by corticosterone is still puzzling. Thus far the effect is unique for corticosterone, a hormone with very weak mineralocorticoid activity in the rat. Efforts to relate the effects of the hormone to induced synthesis of protein are under way. It is anticipated that the isolation of pure protein components of the ATPase system will prove of value here, since it will be possible to demonstrate immunochemically whether the corticosterone stimulation is specific for the protein of the ATPase system.

Mechanism of Uptake and Storage of Catecholamines by Brain and Sympathetically Innervated Tissues

The uptake of catecholamines by an active transport system in the membrane of sympathetic nerves is well established as a mechanism which terminates the physiological effects of these amines. The newly taken up amines are almost instantaneously incorporated into storage granules. The biochemical processes involved in the transfer of catecholamine from the carrier site in the membrane to the storage granule are obscure. In the hope of clarifying this part of the uptake mechanism studies with

norepinephrine storage particles from heart were undertaken.

Methods for the isolation of highly purified amine storage particles from rat heart have been devised and used in studies of the uptake of NE *in vitro*. Uptake by these preparations has been compared with uptake by cruder microsomal fractions similar to those used in other laboratories. It appears that most of the *in vitro* uptake of NE previously ascribed to heart storage particles represents rapid adsorption onto a site that is neither specific for NE nor associated with the storage particle itself. Much of the concentration of isotopic NE ascribed to these particles appears to represent the binding of metabolites. Isolated particles from heart, unlike those from splenic nerve or adrenal medulla, appear to have lost the ability to accept NE by processes related to the *in vivo* uptake.

New Methods of Analysis

An isotopic derivative technique for the assay of secondary amines has been developed and applied to the assay of desmethylimipramine levels in plasma of patients receiving the drug in therapeutic dosage (25 mg per day). The results show that the plasma levels are as low as .030 microgm per ml and that there is a wide individual variability in the rate of biotransformation.

A procedure for the assay of labeled thalidomide in plasma and tissues has been devised. In this method a simple procedure separates unchanged drug from its metabolic products.

LABORATORY OF TECHNICAL DEVELOPMENT

Fluorescence Methods

The development of methods for measurement of the polarization of fluorescence has shown that the Spectrophotofluorimeter (SPF) developed here can be modified to provide fluorescence polarization spectra of sufficient accuracy to permit application of the corrected spectra to the characterization of protein-dye interaction and intramolecular fluorescence interaction.

It has been shown that when the SPF is equipped with a recently developed ultraviolet polarizing filter (Polacoat) set, polarization errors, introduced by the use of grating monochromators, can be eliminated from the measurements. In addition, a relatively simple correcting procedure developed elsewhere has been used to provide the spectral energy versus wavelength correction. The effectiveness of the method is shown by the agreement of our data with values obtained by others in quantum yield measurements and in close agreement of our data with theoretical values of polarization ratios obtainable from dyes in high viscosity solvents. The ability of the technique to indicate the characteristic conformation of the proteins and the interaction of bound dyes was demonstrated by the correlation of the known allosteric behavior of glutamic dehydrogenase (GDH) with results obtained with the fluorescence depolarization method.

Suitable dyes were prepared, purified and methods of dyeing and separating the product were developed and measurements made. Dye enzyme complexes were still highly active and the measurements indicated free rotation of some segments of the molecule. The molecule does not behave as a compact ellipsoid as would be required for determination of axial ratios by fluorescence depolarization previously reported by Weber.

The system of GDH and DPNH studied by the native fluorescence of the tyrosine and tryptophan indicated no heterogeneity of the four DPNH binding sites. The fluorescence polarization of the tryptophan and tyrosine were altered by urea and detergents in agreement with expected behavior. In the observation of the GDH activity, photo-sensitivity of the enzyme required the development of method to reduce the photo-degradation. Published results of these measurements describe the technique of reducing the effect enough to permit accurate measurements. It was also shown that this inactivation by monochromatic light was a phenomenon distinct from the inactivation by x-rays studied elsewhere.

Studies of GDH using quantum yield and fluorescence polarization with dyes, urea, detergents and allosteric regulators are continuing. The binding of acridine dyes to DNA has

been studied elsewhere because these dyes are known to be mutagenic and because it was proposed that the mode of binding is related to the mutagenic activity. The mutagenic effect was explained by Lerman and Luzatti some years ago as owing to coding errors resulting from intercalated binding of the dye. As circular dichroism and spectral titration data were not consistent with the theory, fluorescence polarization studies were undertaken in cooperation with the NIMH physical chemistry laboratory. DNA Acridine Orange (AO) complexes were examined over a range of solution viscosities by utilizing the modified SPF. The bound AO molecules were shown to have degrees of freedom inconsistent with the intercalation theory. Some additional dye-dye interaction which results in a red fluorescence which can be studied with the new red sensitive detectors recently made available for the SPF was also observed.

A microspectrofluorometric system for the study of intracellular tetracycline in micro-organisms has been developed for the Laboratory of Biology of Viruses of NIAID. The system permits satisfactory measurement of the intensity of fluorescence from selected one micron regions of observation. An EMI type 9524S multiplier phototube provides the extreme sensitivity required with a dark current of only 3×10^{-12} A or a 50 to 100 fold signal/noise advantage over the usual 1P21 tube. A fluorescence micro-method for inulin is under development with sensitivity of 5 nanograms. The method offers advantages of simplicity and sensitivity over the currently used colorimetric method. The reaction of 5,5: dimethylcyclohexane 1, 3 dione (dimedon) with fructose in 85% O-phosphoric acid to form a fluorescent product is the basis for the method. Activation at 360 m μ and fluorescence at 410 m μ was established with the SPF and a special purpose filter instrument suitable for reading microcuvettes was constructed. Limitations currently seem to be related to non-uniform fluorescence of the disposable microcuvettes.

Ultramicro Methods

The helium glow photometer has now been developed into a practical instrument that permits the simultaneous analysis of Na and K

content (Picomoles) in submicrogram amounts of kidney tubule tissue to $\pm 5\%$.

During the development it was necessary to determine the mutual interaction of the alkali metals as well as anion effects and develop a suitable diluent to reduce the errors due to these interactions. A diluent containing cesium and phosphate has been satisfactory to "swamp out" potential errors due to enhancement or inhibition of the characteristic emission. The use of a dichroic mirror separates the Na and K emission and high efficiency multilayer interference filters select the lines for measurement by two multiplier phototubes. The photocurrent of each tube is integrated electronically for a specific time to quantify the content of Na and K.

Limitations of the method have been shown to be related to sample handling by comparison with radioactive sodium delivery with the same methods. The use of newly developed pipettes for nanoliter transfer and special coatings of Kel-F have facilitated the necessary transfers.

A micro-pipette for automatic filling and transfer of nanoliter quantities was developed and a description published. The measuring pipette was constructed of quartz and sealed by flame fusion into a larger glass capillary. The use of quartz permits the use of a micro-flame to produce the seal without distorting the 20 to 30 μ ID measuring capillary and the extremely small size permits direct quartz to glass fusion without cracking. Modifications with bulbs for several nanoliters have been constructed; this modification permits the simultaneous observation of both ends of the pipette in the 60x microscope field.

An ultramicro freezing point depression apparatus and method previously described was completed and the results published. Efforts are being continued to optimize the equipment by utilizing commercially developed packages for coolers and for electronic control apparatus in order to derive a practical apparatus that will be useful to other laboratories. One instrument manufacturer has undertaken to produce a commercial instrument and another has indicated interest in doing so. The laboratory prototype has been used to establish the feasibility of the method. A commercial model for evaluation is expected in the near future.

Micro apparatus required for the perfusion and handling of isolated functional sections of renal tubules stimulated the development of special controlled thermoelectric cool stages and controlled temperature chambers. A perfusion pump based on the controlled expansion of a liquid by a programmed, servo-controlled heating schedule delivers from 5 to 50 nanoliters (10^{-9}) per minute from a pumphead containing no moving parts. The head is 1 cm. in diameter by 3 cm. long and is suitable for mounting directly on the manipulator and holding the perfusion pipette with a minimum of total volume or dead space. The design provides freedom from expansion effects due to ambient temperature changes.

Artificial Organs

Blood Oxygenator

Production of our disposable membrane artificial lung undertaken by the Dow Corning Company is proceeding at a slow pace with their development of suitable semi-automatic machinery for mass production of these units. The mass production is reported to be undergoing preliminary test at the moment. It is of interest to note that the General Electric Company has now succeeded in making a pin hole free silicone membrane only 1 mil thick and the final design of our membrane lung is entirely dependent upon the membrane thickness that is to be used. A thin membrane will permit a smaller area to be used with consequent reduction in size and priming volume. It is presumed that the advantages of this new development will be included in the production of a disposable lung. A few preliminary production samples have been received but as these do not represent the final production device they were not considered useful for extended animal testing. Continued development of the pump to be incorporated inside of the artificial lung is being continued with development of suitable valves and pumping ventricles. Information obtained from the biventricular assistors will be utilized in this development.

Blood Pumping

A method of cardiac assistance which provides a squeezing action to both ventricles of the heart is now in the testing stage. In this method a close fitting rubber envelope is placed around both ventricles so that at rest the rubber envelope maintains the heart in a systolic condition. This envelope, suitably constrained by a rigid lexan container, is expanded to the diastolic condition by the introduction of suction between the rubber envelope and the outer lexan shell. The rubber envelope is thus stretched by the unbalance of pressures created by the suction. This rubber envelope is heavy enough so that upon release of suction it snaps back to its normal relaxed resting condition producing the ejection force necessary to pump the blood. Design features of this assistor permit rapid application of the device by application of reduced pressure to the interior of the squeezing or active rubber envelope to suck the heart into place, and, when necessary, the heart can be popped out by similar applications of slightly increased pressure. The use of the close fitting ventricle and the constricting action of the rubber envelope around the ventricle maintains the assistor in position without the necessity for any clamping or tethering device. A lining of silicone jelly helps to maintain the assistor in position and protects the surface of the heart from abrasion. Animal tests have shown that this device remains in place and is capable of supporting the circulation with the ventricles fibrillating for periods up to an hour. When the fibrillating heart was defibrillated normal heart function returned. Testing of the cardiac assistor will be continued with the expectations that it may be of use in supporting the circulation under various clinical emergency situations such as acute heart failure produced by coronary occlusion or for support of the circulation following cardiac surgery when a normal beat cannot be restored. Under these circumstances temporary support of the circulation could be maintained until the heart recovered. It is also conceivable that some such arrangement might be used as a

more permanent installation to support the circulation either indefinitely or until a suitable prosthetic device could be applied, the temporary support providing the delay necessary for the scheduling and production of custommade equipment for the particular individual.

Blood Dialyzer

Utilizing principles of construction and efficient perfusion of a membrane developed with the work on the artificial lung, an effective artificial kidney was produced and tested. A low priming volume and low perfusion pressure with an effective method of stirring the membrane surface film, resulted in a device with very effective near theoretical performance characteristics. As this device incorporated similar production requirements and marketing facilities as the membrane lung, the Dow Corning Corporation is considering the marketing of this device. It is anticipated that this may be suited for home dialysis because of its simplicity and economy. Further experimental work will be continued when a commercial model of this dialyzer becomes available to us for testing.

Fast Reaction Methods

The development of instruments and methods for the study of fast reactions include improvements in time resolution, and reduction of interfering cavitation effects. Several systems which have been assembled and tested permit simultaneous observation of optical density changes and heat production with a time resolution of 2 to 3 milliseconds and thermal resolution of 2×10^{-5} calories. In this system temperature equilibrium is accomplished in 1½ hours, minimal quantities of reactants are required (2 ml per observation) and 99 percent mixing occurs in about 0.4 milliseconds.

A complete instrument constructed by Science Products, incorporating several new design concepts, has been completed for us and provides finer time resolution by driving the pistons of concentric syringes with a pulse of high pressure gas. The rapid acceleration through the mixer into the observation

chamber improves the time resolution to 0.2 milliseconds and a new stopping valve for stopped-flow operation stops the flow in 0.1 millisecond. Reactant requirements have been reduced to 0.5 ml. Fast flow monitoring has been facilitated by the development of a DC magnetic flowmeter with reduced polarization artifact.

High speed mixers required for the efficient mixing of the reactants should minimize thermal and pressure changes at the jets. Reduction of these effects with high speed mixing devices has been proceeding with the construction of a variety of geometrical arrangements of jets and observation tubes which are tested utilizing the gas-operated syringe drivers. Observation of the mixing tube with high speed photography is used to determine the points of cavitation, streaming and turbulence to optimize the design.

Several fast thermal detectors, such as the copper-doped germanium and the strontium-doped barium titanates, have been obtained and are being evaluated with regard to sensitivity and speed of response.

A combination absorption or fluorescence stopped-flow apparatus which permits a reaction time curve to be determined on 0.2 ml of each reagent has been constructed. Time resolution is 2 milliseconds. Reaction mixtures of 5 millimolar aldolase and dihydroxyacetone phosphate have been measured at 245 m μ and 10^{-5} molar bovine serum albumin.

Work is in progress on the determination of heats and rates of reaction of cobaltioxalate ion and ferrous ion for use as a rapid flow apparatus calibration reaction. In order to clarify some points in the theory of hemoglobin oxygenation reactions, thermal measurements of the first oxygen reaction with hemoglobin are being made with the continuous flow thermal apparatus since 0.4 milliseconds time resolution is needed.

A twin cell differential micro-calorimeter has been constructed for the study of the heats of reaction of biochemical and cellular systems. A mixer with a special low heat of mixing has been developed so that 2 to 3 ml of one solution may be mixed with 0.1 to 1 ml of another solution and a total heat of 10 millicalories meas-

ured to 2%. No thermostat is needed for work at any room temperature which is constant to plus or minus 1 degree C. By using external thermostating a temperature range from -20 to 100 degrees C has been obtained in a now available commercial version. For static heat capacity measurements using an electrical heater, stabilities of 5 microcalories have been achieved. Work is being done at present on the heat of reaction of ATPase in red blood cells.

Gas Chromatography

The development of instrumentation for gas chromatography is being continued along several lines utilizing the dependence of velocity of ultrasound on gas composition. The original system which involved vacuum tube circuits has been redesigned and constructed using solid state circuits. The detector volume of a few microliters, high temperature tolerance, non-destructive detection, elimination of radioactivity and predictable physical behavior over a definable range has made the system of sufficient interest that the Micro Tek Corporation has based an instrument on our published results and has independently improved the detector cell to permit a greater temperature range and higher stability. It is expected to appear on the market soon.

This detection system is also directly applicable to analysis of binary gas mixtures without gas chromatography. As the cell dimensions and frequency determine the sensitivity limits, calibration can be made in physical terms without the need for repeated calibrations. It is expected that absolute and permanent calibrations can be made to 0.10%. Apparatus to provide gas mixtures of sufficient accuracy to demonstrate the capabilities of this system over the range of applicability has necessitated the development of a gas mixing pump which will be completed in the next four weeks. Low gas flow measurements for mixing and calibration of similar systems has generally utilized the soap bubble flowmeter but permeability of the film, chemical interaction, or solubility of the gas limits the applicability of this meter to several gases of interest, i.e., NH₃ and CO₂. A dry sealed piston that follows the flow in response to minute changes in pressure has

been tested as a slow flowmeter and a complete instrument which provides flow measurements for flows less than 100 cc/minute with a repeatability of 0.03% has been developed.

Applications of the ultrasound velocity gas chromatography detector have included the analysis of respiratory gases in cooperation with the cardiology branch. One to two ml samples of air are analyzed every 20 to 30 seconds utilizing a 2" silica gel column with nitrogen carrier gas. The method provides the data for rapid repetitive RQ measurements. Another system is being applied to H₂ and CO₂ production for bacterial metabolism studies in a cooperative study with the Laboratory of Microbiology of the National Institute of Dental Research.

New developments in capillary column liquid chromatography as reported by Beyer are limited in sensitivity by the effluent detector system. Ultrasound velocity methods can be applied to liquid systems by utilizing higher ultrasound frequencies and capillary paths. Preliminary tests have indicated a suitable frequency, cell size, and a sensitivity more than adequate for applications to the detection of amino acids separated on liquid capillary columns. Nanogram sensitivities and in 2 to 20 μ l volumes are predictable on the basis of observations in larger cells.

As the dielectric constant of the gas from the gas chromatograph changes with eluted fractions this change has been detected by the use of paired capacitors that alternately determine the frequency of a high frequency oscillator. Continuous measurement of the frequency with a discriminator produces a signal related to changing dielectric constant. The simplicity of the cell and circuitry are the main features. Refinements of the circuit to eliminate the switching noise will be necessary before evaluation can be completed.

Blood Flowmetering

An ultrasonic flowmeter which measures ultrasound velocity upstream and downstream simultaneously has been shown to have superior theoretical capabilities over systems proposed and published elsewhere. There remains some baseline drift to be corrected by the develop-

ment of a suitable phase lock system. Tests on 2 mm diameter crystals separated by 15 mm showed linear, reproducible, bi-directional response. Adaptation of these elements to a satisfactory catheter tip probe remains to be accomplished. A variant of the ultrasound system requiring only a single crystal has had limited success due to the low level of reflected signal from the blood cells and unknown effects due to turbulence in the region of the transducer.

Nuclear magnetic resonance flow methods have been shown to have adequate sensitivity but it is not yet clear how to employ the capabilities of the physical interaction of the magnetic hydrogen nucleus to construct a practical flow metering system for biological observation. Electronic equipment for testing several of the possibilities is nearly complete and will be used with a new magnet to determine the limitations and advantages of the various models.

Mathematics and Computers

1. A computer method of analysis which permits the physical laws governing conduction or diffusion to be expressed in first-order, finite form has been developed. Solutions are obtained in one and two dimensions for rectangular, cylindrical, and spherical geometries without the use of transcendental functions or other conventional mathematical iterative methods. The case of the cylindrical calorimeter with concentric insulating and conducting layers of varying chemical properties and with distributive heat sources arising from biochemical reactions at the center is treated in the transient and steady-state case. Work is being extended to three dimensions and the problem of cell membrane diffusion as well as consecutive and simultaneous chemical reactions. The problem of data handling as it comes from the detector is being solved by digitalizing using a Computer of Average Transients (CAT) or a hybrid control data 3100 computer. Data from the mass spectrometer and stopped-flow reaction apparatus are being programmed.

Retention index systems have been utilized for qualitative component identification in gas

chromatography. Incompletely resolved components exhibit apparent displacement of retention index. According to theory, this displacement results from (1) the simple summation of two unresolved distribution functions and (2) solute interaction. A repetitive analog computer developed in the Laboratory of Technical Development was utilized to investigate the contribution of solute interaction for sample sizes utilized in analytical scale gas chromatography. The results of our investigations demonstrate that for samples smaller than 100 micrograms (as normally employed for analytical scale separations) no significant variation in peak position occurred due to solute interaction. It was further found that the repetitive analog computer is an effective aid in the quantification of partially resolved gas chromatographic peaks(3).

It has been demonstrated mathematically that counterflow systems with active transport out of the ascending limbs in only the outer renal medulla cannot give the known sodium concentration profile in the inner medulla. The importance of these results is that it is possible to rule out a large class of systems as possible concentrating devices.

LABORATORY OF CARDIOVASCULAR PHYSIOLOGY

Net Potassium Changes in the Heart Associated with Altered Contractility

It had previously become clear that if the effects of pharmacologic interventions on the K⁺ balance of the heart are to be fruitfully examined, the hemodynamics of the heart, especially aortic pressure and heart rate, must be maintained constant since alterations in these parameters will affect net K⁺ balance. The isolated supported heart preparation therefore continued to be the experimental preparation of choice. The overall objective of these studies was to gain further insight into the working hypothesis that quantitatively small shifts in K⁺ are importantly related to the contractility changes seen during homeometric autoregulation and certain pharmacologic interventions.

The following table of consolidated data summarized the findings to date.

Intervention	Myocardial Potassium	Myocardial Contractility	Oxygen Consumption
Aortic Pressure			
Increased	—	+	+
Heart Rate			
Increased	—	+	+
Paired Stimulation	—	+	+
Acetyl			
Strophanthidin	—	+	0
Quinidine Sulfate	+	—	0
Pentothal Sodium	+	—	0
Potassium Chloride	+	—	0
Norepinephrine	+	+	0
Calcium Chloride	+	+	0

+ = net K⁺ gain

— = net K⁺ loss

+= increased contractility

-= decreased contractility

+= increased O₂ consumption

0 = no change in O₂ consumption

From these data a consistent pattern of relationships has emerged.

A. Those interventions below the solid line (norepinephrine and calcium) do not influence contractility by way of a net K⁺ loss since the increase in contractility they induce is accompanied by a net gain of K⁺.

B. With the four interventions shown between the solid and dashed lines, a net gain K⁺ is associated with a decreased ventricular contractility and a net loss of K⁺ with an increased contractility. With acetyl strophanthidin, analysis of the fourteen experiments in which this agent was administered showed a good correlation between the dose given and the net K⁺ lost. It will be recalled that small doses of acetyl strophanthidin produce a small contractility increase and large doses produce a large contractility increase. There is, therefore, an important correlation between net K⁺ loss and contractility increase with this agent.

C. Three hemodynamic interventions (including paired stimulation) were made. These are shown above the dashed line in the table. In each instance, a period of increasing contractility occurred as a consequence of the intervention. In each instance a net loss of K⁺ occurred. The range of these net K⁺ losses was comparable to the range of net K⁺ losses observed

with inotropic doses of acetyl strophanthidin.

Studies were continued quantifying the uptake of K⁺ by the heart when KC1 is infused in amounts which elevate left ventricular end-diastolic pressure. The amounts of K⁺ uptake under such circumstances were comparable to those seen to be lost from the heart during the interventions above the solid line in the table.

On the basis of the above results, a reasonable conclusion seems to be that contractility and K⁺ changes are importantly related. The possibility existed, however, that when a rise in left ventricular end-diastolic pressure occurred during infusion of K⁺, that this was produced by altering conduction and therefore synchronicity rather than the contractile fiber event itself. Data have become available relative to the last point. As noted above, with the onset of paired stimulation a marked increase in contractility occurs. A net loss of K⁺ in amounts at least as large as those seen after a large dose of acetyl strophanthidin is also seen. When paired stimulation is terminated, contractility decreases while a net gain of K⁺ is taking place. Observations on conduction before, during and after paired stimulation do not reveal conduction to be altered. These data indicate that if the net K⁺ changes are influencing contractility, they are not doing so by altering conduction.

Further data have been gathered on net K⁺ losses during homeometric autoregulation induced by abrupt increases of aortic pressure while stroke volume and heart rate are held constant. This was done with the objective of having more complete information about the relation between the net K⁺ losses observed and the simultaneous increases in O₂ consumption. It has become clear from the analysis of twenty-two such experiments that the greater the increase in oxygen consumption the greater will be the net loss of K⁺. Some means of ascertaining whether these are simple concomitants or causally related phenomena will be the subject of continuing investigation. With reference to this point an interesting dichotomy is apparent in the table. The pharmacologic interventions investigated uniformly produced net K⁺ changes without oxygen consumption changes. No hemodynamic intervention was observed to produce a net K⁺ change unless a change in oxygen consumption did occur.

It had been reported by others who, in our view, used less rigorously controlled and precise techniques, that insulin and nethalide would dissociate the net loss of K^+ from the increase in contractility when acetyl strophanthidin is given. It was, therefore, felt desirable to repeat such experiments. Our data do not confirm the observation that the preliminary administration of either insulin or nethalide diminishes either the net K^+ loss from the heart or the increase in contractility when acetyl strophanthidin is administered.

Myocardial Oxygen Consumption

Previous work from this laboratory introduced the advantages of relating the oxygen utilization of the heart to the amount of tension developed by ventricular myocardium at least insofar as this is reflected by the area under the systolic portion of the ventricular pressure curve (tension time index). This relationship was seen to hold for any given state of the myocardium.

Two additional bodies of data relative to the general problem of myocardial oxygen consumption in the pumping heart have become available. The first is that with pharmacologically induced increases in contractility in addition to the fall of left ventricular end-diastolic pressure, there is a decrease in the tension time index with no change in oxygen consumption. With agents that decrease contractility, the tension time index is increased with no change in oxygen consumption. In the new contractile state, the oxygen consumption will once again vary with hemodynamically induced changes in the tension time index. There is, in short, a family of curves describing this relationship and any particular curve can be shifted by inotropic influences much as inotropic influences shift the curve relating end-diastolic pressure to stroke work.

The second type of generality that became apparent was that if aortic pressure, stroke volume and heart rate are held constant, inotropic agents can markedly influence the maximum rate of rise of ventricular pressure with no change in oxygen consumption. Only with one of these (norepinephrine) was a significant increase in oxygen consumption observed and

this occurred only when very high doses of the drug were given. Over a large part of its dosage range, left ventricular end-diastolic pressure fell, the duration of ejection shortened, and the maximum rate of rise of ventricular pressure increased with no change in oxygen consumption. These data indicate that when oxygen consumption is increased as the result of inotropic interventions, such an increase cannot be attributed solely to the increase in the maximum rate of rise of ventricular pressure.

It was the complicated geometry of the intact pumping heart that made necessary the use of a tension index instead of a direct calculation of tension. Two approaches have been utilized to effect a transition from this index to a measured value. The first is in a preliminary stage and consists of making simultaneous determinations of pressures, absolute dimensional changes and oxygen consumption. The second approach has yielded a satisfactory body of data from experiments in which the oxygen consumption of papillary muscle, with its relatively uncomplicated geometry, has been examined. An inverse relationship between velocity, shortening and oxygen consumption was seen. The expected lack of correlation between oxygen consumption and external work is also seen in papillary muscles. Isometric studies demonstrated the lack of a direct relationship between changes in initial tension, total tension area, and contractile element work and oxygen consumption. Only the developed tension and developed tension area were found to correlate with changes in oxygen consumption.

Factors Influencing Peripheral Vascular Resistance During Exercise

The possible influence of potassium on the vascular resistance during muscular activity was extensively investigated in the isolated, perfused, canine gracilis muscle. The first phase of this study consisted of studying the net K^+ loss from the muscle associated with the transition from rest to exercise.

The data show that venous K^+ concentrations increase during muscular contraction and that the greater the number of contractions the greater the increase in venous K^+ . The same

relationship holds with arterial inflow occlusion; i.e., the longer the duration of occlusion, the greater is the venous K⁺ level upon restoration of blood flow. Significantly, the degree and particularly the duration of decreased vascular resistance correlated with the degree of muscular activity and the duration of arterial occlusion. Thus, there was also a correlation between the resistance change and the increase in venous K⁺. While this correlation was maintained in all experiments during the period immediately following contractions and following return of blood flow, it was not infrequently observed that resistance could return to control levels and venous K⁺ remain elevated or vice versa. Further, the extent of the K⁺ elevation was quite small (0.2 to 0.8 mEq/L). Infusions of even much larger amounts of K⁺ failed to produce a decrease in vascular resistance that approached that decrease observed with the muscular contraction or arterial occlusion.

Because it was not possible to effect the same degree of vasodilation in the muscle vascular bed by infusing an amount of potassium that produced the same or even higher venous K⁺ level as that associated with exercise and with the arterial occlusion data in view, experiments were designed to determine if a given level of K⁺ might not have a varying vasodilator effect at different O₂ levels.

These experiments yielded data showing that perfusion with high O₂ blood containing low levels of K⁺ (1.0–2.0 mEq/L) will result in an increase in vascular resistance. Perfusion with low O₂ blood (1.0–8 vol.%) containing low levels of K⁺ (1.0–2.0 mEq/L) does not produce an increase in resistance but rather a decreased vascular resistance. Increasing the K⁺ concentration of the perfusing blood, whether of high or low O₂ content, results in a progressive decrease in vascular resistance, the reduction being greater with each increasing K⁺ level with the low O₂ blood than with blood of high O₂ content. Resistances approaching those produced by exercise have not been realized even at extremely high levels of K⁺ when the muscle is perfused with high O₂ blood. It has, however, not been unusual to reach "maximum vasodilation" when the muscle is being perfused with low O₂ blood containing K⁺ in concentrations

well within the venous K⁺ ranges observed as a result of inducing muscular contractions. The data obtained thus far indicate that blood O₂ and potassium levels are importantly related to the degree of tone maintained by the resistance vessels in skeletal muscle and that these factors interrelate and reenforce each other in their influence on peripheral vascular resistance in the transition from rest to exercise.

The powerful vasodilator action of the kallidins has in the past given rise to the suggestion that these substances may contribute to the decrease in vascular resistance associated with muscular activity. Investigation of this possibility was made in the isolated, perfused (constant-flow) gracilis muscle of the dog. In those experiments where carboxypeptidase B was measured in lymph, the lymph was collected from the main lymphatics during gentle exercise of the hind limb of the dog. Infusion of kallidin-9 at 1–4 µg/min directly into the gracilis artery consistently produced a pronounced decrease in resistance which could be effectively blocked by simultaneous perfusion of the muscle with blood containing 15–60 µg/ml hog pancreatic carboxypeptidase B. The vasodilatation produced by muscle contraction, arterial occlusion and lumbar cholinergic sympathetic nerve stimulation was not altered by the presence of carboxypeptidase B. The failure of carboxypeptidase B to block the three interventions could have been due to its absence from perivascular fluid. However, this enzyme, with a molecular weight of 34,300 was found in hind limb lymph following systemic infusion in a ratio similar to that of the plasma proteins. Also, the vasodilatation following occlusion was more prolonged when the occlusion was immediately preceded by muscle contraction than when preceded by kallidin infusion, indicating that the polypeptide was not as stable as the vasoactive metabolites or metabolite released during exercise.

The failure of carboxypeptidase B to alter the decrease in vascular resistance following muscle contraction, arterial occlusion and lumbar cholinergic sympathetic nerve stimulation, the presence of carboxypeptidase B in perivascular fluid, and the greater stability of the exercise factor as compared to kallidin make it unlikely that activation of the kallikrein-kalli-

din system is responsible for these types of vasodilation in contracting skeletal muscle.

Renal Studies

INTRAVASCULAR RECEPTORS IN WATER AND SALT REGULATION. Previous studies from this laboratory have shown that the diuresis associated with acute isotonic iso-oncotic intravascular volume expansion is attenuated by either acute bilateral carotid sinus denervation or acute bilateral cervical vagotomy; sodium excretion is however not influenced. As a continuation of these studies experiments have been undertaken to determine the influence of chronic cardiac denervation upon the diuretic and natriuretic responses to intravascular volume expansion. The renal responses of a group of dogs to volume expansion were determined prior and subsequent to chronic cardiac denervation. The results of the experiments have shown that the absence of cardiac nerves significantly diminishes both the diuretic and natriuretic responses to acute volume expansion apparently independently of changes in glomerular filtration rate. These experiments, taken in conjunction with the earlier acute vagotomy experiments have led us to the position that whereas cardiac vagal afferent nerves appear to modulate free water excretion, cardiac sympathetic nerves may modulate sodium excretion. The cardiac sympathetic nerves concerned appear to be afferent fibers since (1) any influence of sympathetic efferent nerves would be expected to be reflected by changes in filtered sodium secondary to changes in G.F.R. (2) The drug tests used to verify denervation indicate that one of the animals had regained efferent but not afferent innervation (failure to respond to veratramine with bradycardia). This animal continues to show an attenuated diuretic and natriuretic response to acute volume expansion. The chronic denervated dogs usually have completely regained innervation twelve months after the operation. The animals in the present series will be followed to determine if, after this time, the normal natriuretic and diuretic responses to acute volume expansion returns.

It has been reported that section of the vagi in the normal dog undergoing water diuresis is

associated with antidiuresis. It has been assumed that this response was secondary to the section of atrial afferent nerves. In order to determine the influence of extracardiac vagal afferents upon water diuresis, studies were done employing the chronic cardiac denervated dog. Water diuresis was established in this preparation and the influence of bilateral cervical vagotomy determined upon free water clearance, osmolal clearance, inulin clearance and sodium excretion. Bilateral cervical vagotomy was always associated with a reduction in urine flow which was not consistently related to changes in osmolal and inulin clearance or sodium excretion. Free water clearance always declined, the maximum decrease occurring approximately 15 minutes after vagotomy. In some experiments the antidiuresis was well maintained. In other experiments an escape phenomenon was seen in that after the initial reduction, urine flow again increased to or towards the control level. In one experiment supradiaphragmatic vagotomy was done. Section of the vagi at this level had no influence on any of the variables measured while cervical vagotomy produced the characteristic antidiuresis. These experiments support the position that receptors which modulate free water excretion are contained in the pulmonary bed. It is possible that the receptors concerned are osmoreceptors.

To determine further the influence of cardiac nerves on sodium excretion, experiments have been initiated to determine the natriuretic and diuretic response of the chronic cardiac denervated dog to acute saline loading. Although preliminary, the experiments suggest a pattern of response which is different from the dog which has its cardiac nerves intact.

THE RELATION OF THE KALLIKREIN SYSTEM TO RENAL FUNCTION. Earlier studies from this laboratory demonstrated that kallidin is a potent renal vasodilator and produces an increase in glomerular filtration rate, water and electrolyte excretion. It was further observed that these changes could be mimicked by acetylcholine, and that during the intrarenal infusion of acetylcholine the level of an oxytocic substance in renal vein blood increased. To de-

termine the nature of this oxytocic principle it has been necessary to determine the specificity of the method developed in this laboratory for isolating kallidin from blood. Essentially it has been found that the method isolates both the kallidins and angiotensin but does not isolate acetylcholine or serotonin. Chymotrypsin inactivates both polypeptides while trypsin, under controlled conditions specifically destroys angiotensin and kallidinase specifically destroys the kallidins. Presently our studies are directed towards determining the nature of the oxytocic principle found in blood and to determine the nature of increased level of oxytocic principle found in renal vein blood during the infusion of acetylcholine.

Chronic Electrical Stimulation of the Heart

The use of electronic stimuli to control the heart rate chronically has become a practical reality. Recently the use of paired stimuli to produce augmented myocardial contractility has demonstrated again the need for precise analysis of the parameters associated with myocardial stimulation. Analogies taken from work done with nerve stimulation are no longer satisfactory.

Stimulation energy thresholds are determined from the general formula:

$$\text{Energy} = (\text{voltage}) (\text{current}) (\text{time})$$

To study these thresholds comprehensively both acute and chronic electrode implantations were made in dogs, using electrodes of known size and configuration. The available surface area was .055 sq. cm. Acute energy-duration curves demonstrated a minimum of approximately 0.2 microjoules at pulse durations between 0.5 and 1.0 msec. At shorter pulses both voltage and current increase rapidly thereby rapidly increasing energy thresholds and at longer pulses the increased time base increases the energy thresholds, though less abruptly. demonstrated a very similar curve configuration, with a minimum near 3.5 microjoules between 0.5 and 1.0 milliseconds.

Energy thresholds have also been shown to vary with electrode surface area. If the anode surface is enlarged, the total electrode voltage Chronic studies (3 months or more) have

is reduced and current thresholds remain constant. If the cathode surface area is enlarged total voltage also drops (at the same current) but current thresholds are increased. When the current is increased to stimulation threshold levels, the energy threshold has not decreased.

Thus to minimize the energy required for stimulation with these electrodes a small cathode and large anode should be used with a stimulus pulse width of 0.5 to 1.0 msec. Further study to analyze the electrochemical processes which lead to cellular depolarization are in progress.

LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM

The Laboratory of Kidney and Electrolyte Metabolism is devoted to studies of electrolyte and water transport across biological membranes, the role of the renin-angiotensin systems in experimental situations associated with fluid retention and in hypertension, and to cardiac muscle contractility as influenced by a protein system present in mammalian plasma.

Micropuncture Studies in the Kidney

The importance of micropuncture studies in the intact nephron as a means of direct examination of renal transport mechanisms is self-evident. In a previous report a method for pursuing such studies in the proximal nephron of the dog was described. Since that time the method has been improved significantly, and it is now possible to remove repeated samples of tubular fluid from the same proximal tubule throughout the course of a single study. In the past year this technique has been applied to a study of the fractional reabsorption of water (and by inference of sodium since these are reabsorbed by a linked mechanism) in the proximal nephron. It had been postulated in the past on the basis of indirect evidence that fractional reabsorption of tubular fluid in the proximal nephron is relatively constant, uninfluenced by changes in glomerular filtration rate, but is responsive to the action of various diuretic agents. It is known that the injection of saline into intact dogs reduces fractional sodium reabsorption by an unknown mechanism presumably humoral. On the basis of experi-

ments performed in this laboratory utilizing micropuncture technique, it has been concluded in agreement with the earlier inferences that fractional reabsorption of water (and sodium) is in fact virtually constant in the normal animal, and that changes in glomerular filtration rate, whether spontaneous or experimentally induced, result in rapid adjustment, the net effect of which is the maintenance of the constancy of proximal reabsorption. An elucidation of the mechanism of this glomerulotubular readjustment is an important problem which is being actively pursued at the present time.

It has also been observed that the decrease in fractional reabsorption of sodium observed in the intact dog following infusion of isotonic or hypertonic sodium chloride is a result of a significant decrease in reabsorption in the proximal nephron. This firmly establishes the locus of the effect of sodium chloride in this respect. It is of interest that even at the diminished level of sodium reabsorption observed following intravenous saline, acute alterations in GFR, as in the normal animal, do not affect the new steady state fractional reabsorptive rate. In acute thoracic inferior vena caval obstruction, a situation which leads to sodium retention and edema accumulation, micropuncture studies of the proximal nephron were indicative of an increase in the rate of reabsorption of fluid in this segment. No such effect on proximal reabsorption was noted when venous pressure was elevated by obstruction of the abdominal vena cava, a procedure which does not generally lead to the accumulation of edema.

On the basis of preliminary studies, it appears probable that none of the following diuretic agents: hydrochlorothiazide, chlormerodrin, ethacrynic acid, and furosemide diminishes proximal reabsorption. Their effects on sodium reabsorption must, therefore, be exerted at a more distal site in the nephron. In contrast, the infusion of an osmotically active agent, such as mannitol, clearly diminishes proximal reabsorption as had been assumed on the basis of classical clearance studies in the past.

In collaboration with a visiting scientist from Japan, a technique for micropuncture of the otherwise inaccessible intrarenal papilla of the rat was developed. This required operative

removal of a portion of the overlying renal cortex, visualization and mobilization of the intra-renal papilla, and subsequent direct micropuncture of collecting tubules and other structures in this segment. Despite the operative intervention and resultant reduction in renal mass, the kidney functions in a qualitatively relatively normal manner in that the elaboration of the hypertonic urine (less so than from the unoperated kidney) was observed. Furthermore, was possible to establish that, as in species in which the papilla was not intrarenal and therefore accessible to direct examination, the osmolarity of the tubule fluid in collecting ducts increases progressively towards the tip of the papilla. Finally, a small potential difference of 11 microvolts, tubule lumen negative to interstitium, was also observed.

Isolated Tubules *in Vitro*

For a number of years this laboratory has been engaged in an analysis of electrolyte exchange within cortical slices of rabbit kidneys utilizing classical isotope techniques. The difficulty of interpretation of the results inherent in the heterogeneity of the tissue and the influence of non-cellular compartments led to development of a technique to perform similar studies in suspensions of isolated cortical tubules *in vitro*. The results of these studies have been reported in the past and, though the removal of an interfering extracellular phase in the newer preparation minimized some of the theoretical difficulties in interpretation, it was still impossible to relate the observations to transcellular fluxes directly for a number of reasons, including the heterogeneity of the tubule fragments within the suspension. In the past year a major improvement in technique has been developed which permits the perfusion of single isolated tubule fragments *in vitro*. The fragments are dissected by hand from fresh rabbit kidneys without prior treatment. It was possible to analyze the water and electrolyte content of fragments of glomerulus, proximal convoluted tubule, proximal straight tubule, thick ascending tubule and collecting tubule, and it was observed that the potassium content of these segments closely approximates that observed in the past in both kidney slices

and suspension of renal cortex, evidence, in part, that the tubules are viable since they are capable of maintaining relatively normal concentration gradients for potassium. Similarly, the content of sodium and water of the proximal tubule approximates that in slices and suspensions. The sodium content of the other segments has not yet been estimated with certainty. Isotopic exchange utilizing K^{42} and NA^{22} has also been performed.

In the studies alluded to above, and reported in a previous Annual Report, a minimum of two tissue compartments of each ion had been observed in the suspension. It appears likely, however, on the basis of the present studies, that, insofar as potassium is concerned, these compartments may represent different tubule segments rather than compartments within the same cell. On the other hand, the kinetic compartmentalization of sodium observed in the tubule suspension may, in fact, be due to intracellular heterogeneity, though this has not yet been established with certainty. However, at least two compartments, one rapid, the other slow, have been observed in proximal isolated tubule. Since the former is uninfluenced by external sodium concentration exchange diffusion may be excluded (see below).

Having established that such isolated tubules are capable of maintaining essentially normal electrochemical gradients for sodium and potassium, micro-perfusion of isolated tubules was performed. This required utilization of a specially developed concentric double-barreled micropipet. It has been established that the proximal tubule accumulates chlorphenol red as does the intact nephron *in situ*. Furthermore, PAH, as in the living kidney, is actively transported from an outside bathing medium to a PAH-free perfusing fluid (the luminal fluid). Further evidence of the adequacy of the technique, as well as the viability of the preparation, is a demonstration of its impermeability to inulin and its ability to concentrate inulin within the lumen in consequence of net water reabsorption across the cell. Thus, it is now possible to evaluate agents which alter water permeability of the nephron. A direct effect of vasopressin on water permeability was observed in perfused segments of collecting tubule. Both vasopressin and its presumed

intracellular mediator, cyclic AMP, increased the water permeability of the collecting tubule as estimated by the use of tritiated water. Vasopressin also increases the net movement of water along an osmotic gradient as is assumed to occur in the intact kidney.

Chloride fluxes in both separated renal tubules and isolated proximal convoluted tubules have also been studied. It has been observed in the suspension studies that the rate of chloride exchange is significantly less than that of sodium, presumptive evidence that earlier estimates of transcellular transport of sodium chloride reported from this laboratory were erroneously high. They were originally based only on the very rapid rate of sodium exchange noted at that time.

In neither suspensions of tubules nor isolated proximal tubules is there evidence for a purely passive distribution of chloride. For example, although the addition of the cardiac aglycone strophanthidin, a supposed inhibitor of active sodium transport, results in an increase in the tissue chloride content in the isolated tubule and in the suspension, no significant effect on cell chloride content is exerted by alterations in the concentration of extracellular potassium. Thus, the calculated Donnan ratios for potassium and chloride differ at all medium potassium concentrations, a situation unlike that in skeletal muscle in which Donnan equilibrium is maintained at all but the lowest external potassium concentrations.

Toad Bladder

It had been proposed in a previous report that vasopressin increases the permeability of the toad bladder and renal tubule to water by stimulating the production of an intracellular intermediate, cyclic 3', 5'-adenosine monophosphate, in the tissue. The thesis was based on physiological studies summarized in detail in the earlier reports in which vasopressin, cyclic-AMP, and theophylline, which prevents the degradation of cyclic-AMP, have similar effects on sodium and water movement in toad bladder. Direct evidence in support of the thesis is provided this year by the demonstration that the concentration of cyclic-AMP in bladder tissue is significantly increased by

preincubation of toad bladder with either vasopressin or theophylline or a combination of both agents. The mechanism of action of cyclic-AMP on the biochemical processes within the cell as well as the nature of the alteration of the structure of the membrane which permits more rapid water flow and sodium transport is currently under investigation.

An attempt at characterization of the effect of vasopressin and its intermediate (cyclic-AMP) on biochemical processes in the toad bladder has been initiated. Earlier studies in this laboratory had demonstrated that vasopressin and cyclic-AMP stimulate oxygen consumption and glycogen utilization by the toad bladder incubated in Ringer solution containing sodium. No effect on oxygen consumption was observed when choline was substituted for the sodium in the Ringer solution. In addition it was noted that vasopressin or cyclic-AMP stimulates phosphorylase activity in toad bladder regardless of the sodium content of the Ringer solution. In contrast to the results of other workers, it has also been shown that a number of metabolic inhibitors, including dinitrophenol nitrogen, iodacetic acid, etc. interfere with the effects of vasopressin on water and sodium movement across the toad bladder, whereas certain other inhibitors do not. The results thus far suggest that basal sodium transport and stimulation of sodium transport by vasopressin are not dependent on an intact glycolytic pathway but require an intact tricarboxylic acid cycle. On the other hand, the effect of the hormone on water movement does not appear to be closely linked either to glycolysis or to the tricarboxylic acid cycle. It appears to be intact if either pathway is available. This is based in part on the observation that a specific inhibitor of the Krebs cycle which limits the sodium response does not interfere with the water response to hormone, whereas the inhibitory effect on the water response of at least one of the glycolytic inhibitors can be reversed by the addition of substrate, namely pyruvate.

A more precise approach to the metabolic effect of vasopressin has been undertaken by measuring the effects of the hormone on the pool sizes of various biochemical intermediates and co-factors in bladder. Thus far it has been observed that vasopressin increases the concen-

tration of glucose-6-phosphate and certain other glycolytic intermediates; this correlates well with the results of studies on phosphorylase activation mentioned earlier. No change was observed in the concentration of adenosine triphosphate and adenosine diphosphate.

The effect of a naturally occurring fatty acid, prostaglandin, which has been isolated by others from a number of sources including human vesicular plasma, on water permeability of the toad bladder has been examined. Prostaglandin while itself without effect on permeability, in extremely low concentrations, is capable of significantly inhibiting the permeability response to antidiuretic hormone and theophylline, but not to that of cyclic-AMP. Whether this indicates a physiological regulatory role of prostaglandin on water movement *in vivo* is unknown.

The influence of changes in the cation composition of the bathing medium and of the toad bladder cell itself on vasopressin responsiveness is also under study. Thus far it is apparent that removal of potassium from the blood surface of the toad bladder causes a marked depression of sodium transport and the effect of vasopressin on sodium transport within a relatively short time. In contrast, the effect of the hormone on water permeability is not altered until a considerably longer period, approximately one hour, has elapsed. The diminished permeability response with respect to water appears to be best correlated with a decrease in the cell content of potassium. Thus, though strophanthidin, a cardiac aglycone, inhibits sodium transport immediately in toad bladder, it does not interfere with the water response to ADH until a significant decrease in potassium content of the tissue develops. This effect is rapidly reversible upon addition of potassium to the bathing medium.

Finally, in an effort of dissociate the effect of vasopressin and its analogues on water and sodium movement, the effects of these compounds in the same bladder on simultaneously determined net water movement and short circuit current (an estimate of net sodium transport) were examined. It was not possible to define clearly any important differences between the various analogues studied with a single exception. Arginine vasotocin appeared to yield less

stimulation of sodium transport than enhancement of water permeability at a dose at which other compounds yielded equivalent effects on the two functions.

Electrolyte Transport Across Red Cells

The studies of the characteristics of electrolyte transport across red cell membranes of a variety of species are continuing. As in the past, hemolyzed preparations of red cells (so-called ghosts) in which it is possible to vary the intracellular environment at will, have been used to advantage. On the basis of earlier work in this and other laboratories, it had been assumed that transport of ions, as measured by unidirectional fluxes, can be operationally separated into at least three distinct components; active transport, that is, transport against an electrochemical gradient, passive diffusion, movement downhill along the electrochemical gradient, and so-called exchange diffusion. This last is thought to be a one to one exchange of an ion—for example, sodium for sodium or potassium for potassium. Exchange diffusion in which no net movement can be accomplished is experimentally defined by measuring the change in the unidirectional flux of an ion as a function of the concentration of that ion in the receiving medium. Thus, that component of sodium efflux, for example, which is eliminated when sodium is removed from the external medium, is said to represent exchange diffusion.

Recent studies have cast considerable doubt on this operational definition. Since exchange diffusion is symmetrical, any manipulation which modifies one of the unidirectional fluxes should exert a similar effect on the other. In many situations this has not turned out to be the case. Thus, although a decrease in external sodium diminishes sodium efflux, a predictable change in sodium influx does not occur in association with changes in the intracellular sodium content. The sodium sensitive efflux also appears to be energy dependent. Cells depleted of metabolic substrate lose the ability to pump sodium and potassium but still retain the sodium-sensitive component of efflux; this too disappears after further metabolic depletion. Also, a differential effect of substrate on the

active transport process and the sodium sensitive process has been discerned. The addition of inosine to cells devoid of both processes activates the sodium-sensitive component of efflux, but does not affect that dependent on external potassium. In contrast, adenosine reactivates both forms of transport.

Studies of potassium efflux have similarly shown that the fraction of efflux dependent on external potassium does not satisfy the criteria for exchange diffusion. Thus, glycosides inhibit the potassium-sensitive efflux without a symmetrical inhibition of potassium influx. The two nucleosides, adenosine or inosine, inhibit the potassium-sensitive efflux but have no effect on influx. It is therefore clear that the operational definition of sodium and potassium exchange diffusion as those components of flux dependent on the trans concentration of the cation requires revision. The sodium-sensitive efflux may represent a form of active sodium transport which does not require potassium.

One interesting aspect of the problem has been disclosed in a study of red cells from patients with cystic fibrosis, since the electrolyte and water composition of these cells is indistinguishable from that of normal ones. No potassium-sensitive potassium efflux component is discernible.

Dog red cells which differ from human and many other animal red cells in that they contain a high rather than low concentration of sodium and a low rather than high concentration of potassium have also been studied. These cells appear to lack a cardiac glycoside-sensitive cation pump. Furthermore, the permeability to both sodium and potassium are remarkably sensitive to changes in cell volume. Shrunken cells are highly permeable to sodium and relatively impermeable to potassium, whereas when swollen these same cells have a low permeability to sodium and a high permeability to potassium. These effects are also metabolically linked in that energy-depleted cells do not reveal the volume-induced changes in permeability unless additional substrate is provided. The steroid, aldosterone, in confirmation of work reported by others, retards the influx of Na^{2+} into dog red cells—an effect which is demonstrable only in the presence of plasma,

indicative of the necessity of some plasma co-factor for the phenomenon.

The relationship between the rates of glycolysis and cation transport in human red cells has also been re-examined. In the past it had been observed that glycosides which interfere with or inhibit active transport do not simultaneously alter the steady state production of lactate, indicative of an absence of effect on glycolysis. This observation was made in utilizing normal red cells. In contrast, stored cells, in which some degree of substrate depletion is achieved, respond to glycosides by a further reduction in lactate production. On the basis of preliminary observations, it appears that this effect is related to the altered intracellular sodium and potassium content, and that sodium transport and lactate production are linked. Thus, high sodium cells under certain conditions transport sodium more rapidly and simultaneously increase lactate production. The effect is analogous to that noted in tissues such as kidney and frog skin in which the level of oxygen consumption appears to be regulated by the rate of sodium transport.

Renin-Angiotensin Aldosterone System

An important segment of the laboratory is devoted to an intensive and long-term study of the role of the renin-angiotensin-aldosterone system in fluid accumulation (experimental heart failure) and hypertension. In the past they have provided important evidence that the renin-angiotensin system in the kidney is involved in the regulation of aldosterone secretion by the adrenal. Further evidence has been accumulated in the past year concerning the role of this system in congestive heart failure, hypertension and other experimental states.

Hypertensive dogs have been studied in an effort to determine the relationship of plasma renin concentration and aldosterone secretion to blood pressure. For this study two groups of experimental animals, one with acute experimental renal hypertension, the other with chronic experimental renal hypertension, has been compared to normal controls. It was noted that an elevation of plasma renin and increased rate of aldosterone secretion occurred only during the first few days of hypertension in an-

imals which ultimately developed the chronic form of the disease. In contrast, in dogs with acute malignant hypertension plasma renin remained elevated throughout the entire course of the disease which lasted for as long as 13 days. In these, aldosterone secretion was also generally elevated. Arterial hypertension developed in both groups approximately two day following application of a Goldblatt clamp to the renal artery.

In another study directed at evaluating the role of renin hyperaldosteronism in renal hypertension, an attempt was made to determine whether renal artery constriction would, as in normal animals, produce hypertension and a further increase in plasma renin in animals with experimental secondary hyperaldosteronism. The animals in the control phase of the study all had increased plasma renin content and hyperaldosteronism, but of 16 dogs with secondary aldosteronism due to thoracic caval constriction, only 4 developed hypertension following renal artery clamping, and plasma renin rose to higher levels in only 2 of these animals. Of 6 sodium-depleted animals with secondary hyperaldosteronism, in only 2 did hypertension develop following clamping and in none did plasma renin increase. Sodium depletion of 5 dogs with renal hypertension, on the other hand, produced a "normal" response in that plasma renin increased.

Sodium depletion is known to result in enhanced rates of aldosterone secretion and increased plasma renin concentration. However, in addition, evidence has accumulated to indicate that an associate increase in corticosterone secretion does not take place. This appears to be a consequence of an inhibitory effect of the intact pituitary since both aldosterone secretion and corticosteroid secretion are augmented following sodium depletion in hypophysectomized animals. The enhancement in aldosterone secretion is not as great under these conditions as in non-hypophysectomized dogs indicative of an important supportive role of the anterior pituitary.

A delay in the metabolism of aldosterone may play a significant role in certain situations associated with hyperaldosteronism. In order to evaluate this possibility, the disappearance of tritiated aldosterone from peripheral plasma

was studied in a number of experimental conditions. It was noted that the slow component of the disappearance curve was prolonged and the so-called metabolic clearance rate diminished in dogs after hemorrhage, following hypophysectomy, and in experimentally induced low output heart failure. Aldosterone metabolism, on the other hand, was not detectably altered in dogs with high output failure or during sodium depletion. It is assumed, therefore, that hyperaldosteronism seen in the first three conditions is in part at least a consequence of the decrease in the rate of the degradative process. Since there is negligible extrahepatic clearance of aldosterone from plasma, it was of interest to study the characteristics of hepatic extraction of the steroid in a series of animals. It was concluded that the hepatic mechanism for the metabolism of aldosterone is flow limited rather than limited by the rate of hepatic metabolism, since virtually complete extraction of aldosterone was accomplished by the liver in normal dogs as well as in those following hemorrhage, sodium depletion, hypophysectomy, and during severe secondary congestion of the liver due to thoracic caval constriction. Finally, it was observed that hepatic blood flow is diminished in those situations in which aldosterone metabolism is delayed.

One of the more puzzling problems relating to aldosterone is the increased sensitivity of the kidney to salt-retaining adrenal steroids in certain states associated with experimental or spontaneous fluid retention. Thus, the animal with a constriction of the thoracic vena cava is exquisitely sensitive to desoxycorticosterone and the salt retention produced by this agent persists throughout the course of a prolonged period of administration. In contrast, the normal dog, as does man, accumulates fluid only transiently and despite continued administration of large doses of desoxycorticosterone, appears to "escape" from the salt retention effects of the hormone. This laboratory has suggested in the past that the escape phenomenon may be regulated by, or dependent on, another circulating hormone. In an effort to determine the validity of this thesis, first by exclusion and then by direct demonstration, the following studies were performed: The role of a decrease in glomerular filtration rate in the

development of the increased sensitivity of the renal tubules to the salt-retaining steroid was eliminated by demonstrating that a "normal" escape from desoxycorticosterone occurs in unilaterally nephrectomized animals in which filtration rate is reduced by application of a renal arterial clamp.

Of greater significance has been the recent observation that cross-transfusion of blood from "DOCA-escape" sodium-loaded dogs to normal recipient animals resulted, in six successful studies, in the development of some degree of natriuresis in the recipient animal. Although an increase in filtration rate and renal blood flow in the recipient animal usually occurred, this was not a uniform finding and, in some there was either no change or a fall in GFR. Of significance is the fact that natriuresis occurred in all regardless of the changes in filtration rate. It seems likely, therefore, that a humoral factor may have been responsible for these results, although this cannot be concluded with certainty as yet.

The biochemical synthesis of aldosterone is thought to involve desoxycorticosterone as a precursor for corticosterone, the latter, in turn preceding aldosterone in the synthetic chain. It is of interest, therefore, that the infusion of angiotensin, a known stimulator of aldosterone and corticosterone secretion, did not increase the secretion of desoxycorticosterone from the adrenal gland of the dog. Similarly, desoxycorticosterone secretion is not increased in dogs with experimental congestive heart failure, though the secretion of aldosterone and corticosterone are.

The effects of the administration of the diuretics, organic mercury and chlorothiazide, on plasma renin and aldosterone secretion were also studied. Following diuresis due to both of these compounds, plasma renin and aldosterone secretion increased. In the case of chlorothiazide the augmentation of plasma renin and aldosterone was prevented by administration of intravenous saline at a rate in excess of the drug-induced sodium loss. It was concluded in agreement with other investigators that renin release and subsequent enhancement of aldosterone secretion following chlorothiazide are the consequences of prior salt loss and not a direct effect of the compound. Although similar

conclusions may apply to the effect of mercury, the results of replacement of sodium were not uniform, and in some dogs this did not prevent the renin and aldosterone changes.

Finally, the renin-angiotensin system has been examined in other animals. On the basis of preliminary results, it appears that a system which functions in a manner similar to that in the dog and in other mammals is present in the very primitive opossum. Similar studies are in progress in the American bullfrog. In the white laboratory rat the principal glucocorticoid secreted by the adrenal is corticosterone; this is not the case in the golden hamster. In the latter cortisol is the major glucocorticoid present in adrenal blood, and furthermore, detectable amounts of aldosterone and corticosterone are also present.

Cardiac Muscle Contraction

It has been previously reported that cardioglobulin-C, one of the three fractions of the inotropic cardioglobulin system, contains calcium. It was also suggested that cardioglobulin-C may, in part, act as a carrier for the calcium necessary for the induction of shortening of muscle contractile protein. In the past year it has been possible to determine with considerable accuracy the calcium content of ashed cardioglobulin-C samples. On the basis of an analysis of the amount of cardioglobulin-C necessary for a maximal inotropic effect in frog heart, the amount of cardioglobulin calcium bound to frog heart under these circumstances has been calculated. This figure has been compared to a similar estimate based on radioactivation induced by cardioglobulin in the absence of external calcium involves the movement of car- consistent with the view that muscle contraction induced by cardioglobulin in the absence of external calcium involves the movement of cardioglobulin-C calcium in an amount which is comparable to the influx of free calcium reported by others to be associated with a single frog heart contraction.

In the earlier report the results of cardioglobulin assays in the plasma of patients with so-called idiopathic heart failure have been summarized. Approximately 50% of these patients

had abnormally low cardioglobulin content, and this was considered to reflect either a diminution in cardioglobulin-C or cardioglobulin-A. Utilizing a more precise assay system developed in the past year this difference between the control and experimental groups with idiopathic heart failure was not observed. This result has stimulated a reinvestigation of the entire problem.

If the cardioglobulin system is a naturally occurring system in the mammal and its binding characteristics to heart muscle are similar to that observed in the isolated frog heart, it should be possible to demonstrate the localization of cardioglobulin-C on the surface of mammalian heart muscle. With this in mind, an immunofluorescent antibody study has been initiated. Antibodies to rat cardioglobulin-C were induced in the guinea pig and were then added to a number of tissues from the rat. Localization was determined by standard immunofluorescence techniques. It was assumed that localization would indicate the site of endogenous cardioglobulin-C in the rat. That antibody to cardioglobulin-C was in fact formed in the guinea pig was proved by the ability of the guinea pig antiserum to inhibit the inotropic effect of cardioglobulin-C on frog heart as well as the demonstration of its binding to cardioglobulin-treated heart, but not to untreated heart. Although antisera did in fact "stick" to rat heart surface, it also adhered to skeletal muscle and to a number of other tissue sites including basement membranes of the renal glomerulus, renal tubules, etc. Binding could be prevented by preliminary passage of the antisera through cardioglobulin-treated frog heart as well as by exposure to washed homogenized rat kidney.

The appropriate interpretation of these findings is not yet clear.

LABORATORY OF METABOLISM

The work of the Laboratory of Metabolism will be summarized here by Section. Members of the three Sections continue to collaborate productively on a number of problems, but the bulk of the work in each group stands independently and is so reviewed.

Section on Metabolism

Mobilization and Utilization of Free Fatty Acids

METABOLISM OF ADIPOSE TISSUE STUDIED IN VITRO. The first direct evidence in cell-free preparations for the presence of two independent systems for activating and for inactivating adipose tissue lipase, respectively, has been obtained. Demonstration of this was made possible by a large difference in pH optimum for the two processes. The activating system was prepared in a particle-free fraction and some of its properties were determined. Through the rapid operation of these systems, it is now clear that the adipose tissue can rapidly adjust its rate of mobilization of free fatty acids (FFA) by inter-converting active and inactive forms of the hormone-sensitive lipase. The studies in cellfree preparations establish that the process is not dependent upon synthesis of new enzyme protein but, like the activation of phosphorylase, represents conversion of an inactive to an active form of the enzyme.

The cortisol stimulation of adipose tissue lipase activity has been confirmed. Further studies show that the use of serum as an incubation medium strikingly enhances the cortisol effect. Dialyzed serum on the other hand is much less effective, but simple addition of glucose restores its effectiveness. It is concluded that maximum cortisol stimulation requires both a non-dialyzable fraction of serum and glucose. It is hoped that further studies along this line, since they relate to a specific and readily investigated system (i.e. activation of lipase), may increase our understanding of the still mysterious mechanism of action of cortisol.

Techniques for study of adipose tissue with the electron microscope are being developed. Earlier studies in this laboratory showed the presence of very small, electron-dense particles in homogenates of adipose tissue. These particles have now been demonstrated in the cytoplasm of intact fat cells, and preliminary studies indicate that the distribution of these is altered by exposure of the tissue to lipolytic hormones. These particles are isolated along with the fat layer at the top of the tube after centrifugation of homogenates of adipose tis-

sue. This layer contains most of the lipase activity of the cell and also most of the proteolytic activity involved in the degradation of peptide hormones. Further studies are planned to determine whether these enzymatic activities actually reside in the described particles.

EFFECTS OF PROSTAGLANDIN ON FFA MOBILIZATION. It was first shown in this laboratory last year that prostaglandin E₁ (PGE₁) is remarkably potent, both in vitro and in vivo, in counteracting the fat-mobilizing action of catecholamines. Concentrations as low as 10⁻⁸ M in vitro are effective in counteracting stimulation of FFA release by catecholamines present in 2 to 5 times this molar concentration. It has not been shown that in unanesthetized dogs, PGE₁ can profoundly depress plasma FF levels when given in single large doses intravenously. Investigators at the Karolinska Institute in Stockholm have observed *increases* in plasma FFA levels in man during constant intravenous infusions of PGE₁. Unanesthetized dogs do not show this response under the same experimental conditions. It will be of interest to determine whether or not human adipose tissue in vitro responds differently from rat adipose tissue.

The findings in these PGE₁ studies are difficult to reconcile with the Randle hypothesis regarding competition between FFA and glucose for utilization by peripheral tissues. PGE₁ caused profound drops in FFA level with either no change or a slight increase in plasma glucose level. Similar observations have been made in man during intravenous administration of nicotinic acid. If high levels of plasma FFA suppress glucose utilization, one might expect the plasma glucose levels to drop when the plasma FFA level is rapidly dropped to very low levels. Definitive conclusions will require measurement of glucose turnover using radioisotopes.

UTILIZATION OF FFA BY ISOLATED CELL SUSPENSION. New insights into the basic mechanisms and the kinetics of FFA utilization have been obtained. The Ehrlich ascites tumor cell was chosen as a readily available model system for studying isolated individual cells with regard to FFA uptake and utilization. As reported last year, the initial transfer of FFA

from FFA-albumin complexes to the cell surface takes place with remarkable rapidity. The mechanism appears to depend upon prior dissociation of the complex and then uptake of the FFA anions. It has now been shown that the subsequent utilization, both for oxidation and esterification, occurs at a rate that correlates with the total "load" absorbed initially onto the cells. The latter is shown to be a function of the molar ratio of FFA to albumin in the medium to which the cells are exposed. From these studies, it is now clear that overall FFA utilization will be dependent ultimately on this molar ratio in the bathing medium. These results in the model system are consistent with a number of observations made in perfused mammalian tissues and in tissue slices.

The binding sites on the cell do not appear to be specifically "assigned" to individual long-chain fatty acids. The total binding of FFA is approximately the same whether a single species of fatty acid is used or whether a mixture of different fatty acids is used. If these results can be extrapolated, they imply that the differences in uptake of different fatty acid species that have been observed in other studies are to be attributed not to differences in binding, but rather to differences in K_m of the enzymes responsible for subsequent metabolic transformations of the FFA taken up initially.

EFFECTS OF FFA AND OF CATECHOLAMINES ON MYOCARDIAL ENERGY METABOLISM. Studies reported last year showed for the first time that perfusion of the isolated rat heart with a medium containing concentrations of FFA increases the total oxygen consumption of the myocardium. It has now been shown that a non-metabolizable fatty acid analogue (3,3,12,12-tetramethylmyristic acid) does not cause this stimulation of oxygen consumption, suggesting that it is not the long-chain fatty acid per se, but rather some subsequent metabolite (e.g. fatty acyl CoA) that is the effective form. This analogue has been shown to be very poorly incorporated into esters, and it is not oxidized at all.

The percentage increment in oxygen consumption caused by FFA was much smaller in the beating heart than it was in the heart arrested by perfusion with high concentrations

of potassium. This suggested the possibility that the pattern of electron transport supplying energy for what might be called the "basal metabolism" of the myocardium might be different from that prevailing in the beating heart. Recent studies with oligomycin are consistent with this possibility. Oligomycin rapidly causes degeneration of the beating heart with arrest within 5 or 10 minutes. On the other hand, oligomycin under the same conditions does not significantly depress oxygen consumption of the potassium-arrested heart. Even in the presence of oligomycin, FFA stimulate oxygen consumption of the arrested heart. It has been shown in Chance's laboratory that oligomycin blocks ATP formation but does not prevent utilization of high energy intermediates for certain cell functions, such as ion transport. The present results suggest, then, that the "basal metabolism" of the arrested heart preparation may depend to a large extent on reactions not linked obligatorily to ATP formation.

Evidence was obtained to show that in addition to its chronotropic and inotropic effects, epinephrine stimulates oxygen consumption by an independent mechanism in the arrested heart preparation. This increase in oxygen consumption of the arrested heart was accompanied by an increase in the rate of release of glycerol to the perfusing medium and an increase in the FFA concentrations within the myocardium. This raises the possibility that the underlying mechanism may be related to that responsible for the increased oxygen consumption produced by perfusing the heart with a medium of high FFA concentration.

Finally, recent studies show that octanoate and β -hydroxybutyrate stimulate oxygen consumption in the arrested heart even though they do not cause significant stimulation in the beating heart. Because the absolute increment might be difficult to demonstrate significantly in the beating heart, it cannot be concluded that the mechanism of the effect is different in the two preparations. The importance of this observation is that it suggests that there may not be a basic difference between long-chain and short-chain fatty acids in the system under investigation.

CLINICAL STUDIES OF FFA AND TRIGLYCERIDE TURNOVER. In collaboration with Dr. Mones Berman, an intensive study of the kinetics of FFA turnover and of the utilization of FFA for synthesis of plasma triglycerides has been undertaken, utilizing computer techniques for analysis of results. The results suggest (a) that there is a large pool of fatty acids in rapid dynamic equilibrium with the plasma FFA, and (b) that fatty acids entering the liver are mixed with a large pool prior to discharge into the plasma compartment again as triglyceride fatty acids. From studies of the rate of appearance of radioactivity in plasma triglycerides, it is possible to estimate the turnover of the latter.

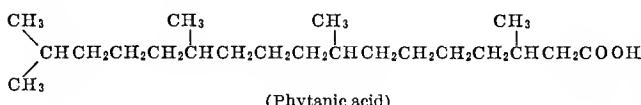
In normal individuals, the half-life of the total low-density lipoprotein triglyceride appears to be about 2 hours. The validity of the model was tested in a patient with fat-induced hyperlipemia, and here the half-life was found to be greater than 20 hours.

In order to test the validity of this approach more directly, a method has been devised for the *in vitro* labeling of human plasma lipoprotein triglycerides. Preliminary animal studies suggest that the method for *in vitro* labeling does not alter the native state of the lipoprotein and that the initial rate of removal of lipoprotein labeled in this way parallels the rate of removal of biologically labeled lipoproteins. This method will be used to obtain by direct means values for the extent to which triglycerides return fatty acids to the FFA pool. The method promises to be extremely valuable in connection with studies of triglyceride turnover in hyperlipemic states.

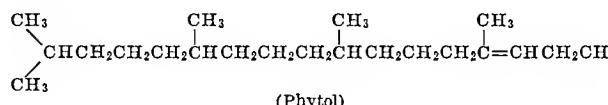
Metabolic Studies of Refsum's Syndrome, a new Lipid Storage Disease

Heredopathia atactica polyneuritiformis (Refsum's syndrome) is characterized by retinitis pigmentosa, hypertrophic peripheral neuropathy, cerebellar ataxia, elevated levels of protein in the spinal fluid, electrocardiographic abnormalities and, in some cases, by sudden death. The disease is familial and the pattern of inheritance suggests a recessive mode. In 1963, Klenk and Kahlke in Germany first reported a remarkable accumulation of phytan-

ic acid (3,7,11,15-tetramethylhexadecanoic acid) in the blood and tissues of these patients:



The tetra-terpenoid structure of this acid suggested that it might be formed by extension of the pathway for biosynthesis of cholesterol. Addition of a fourth isoprene unit to farnesyl pyrophosphate could generate the branched-chain carbon skeleton which might be further modified to form phytanic acid. The second possibility considered was that phytol, a normal constituent of the chlorophyll molecule, might be absorbed and converted to phytanic acid:



Because we were unable to identify a case of Refsum's syndrome in the United States, despite many inquiries at a number of university centers, we got in touch with Professor Sigvald Refsum in Oslo and established a collaborative program with his group at Rikshospitalet. Clinical experiments with labeled substrates prepared here are being carried out in Oslo, and isolation and analysis of metabolites is being conducted on samples shipped to us. Concurrently, the group in Oslo is carrying out ancillary studies at Rikshospitalet.

Studies after intravenous injection of mevalonic acid-2-C¹⁴ failed to reveal significant incorporation into plasma phytanic acid, even though incorporation into plasma cholesterol occurred at a normal rate.

Meanwhile, studies carried out here demonstrated for the first time that orally administered radioactive phytol is readily absorbed by the rat and converted to phytanic acid. By feeding large daily doses of phytol (5% in the diet) it was possible to produce striking accumulation of phytanic acid in the plasma and liver of the rat. Phytanic acid accounted for over 20% of the total fatty acids in plasma and liver. Using C¹⁴-phytol, it was shown that the compound is well absorbed, primarily by way of the lymph, and that over 30% of the ab-

sorbed dose was converted to $C^{14}O_2$ in the first 24 hours. In other words, the normal animal has a considerable capacity to metabolize this branched-chain structure. This is consistent with the fact that very large daily intakes were necessary in order to produce accumulation of phytanic acid. The amounts of *phytol* accumulating were rather small, indicating that *phytol* is very readily converted to the acid by reduction of the double bond and oxidation of the alcohol function. It was shown that feeding *phytol* to germ-free rats also caused accumulation of phytanic acid, proving that the conversion was not dependent upon the action of intestinal micro-organisms.

With these results in experimental animals as a basis, C^{14} -*phytol* was fed to normal control subjects in the Clinical Center and to a subject with Refsum's syndrome in Oslo. Both the normal subject and the patient absorbed more than 80% of the tracer dose. The normal subject oxidized 27% of the administered dose to $C^{14}O_2$ in the first 12 hours. In contrast, the patient with Refsum's syndrome oxidized less than 3% to $C^{14}O_2$ in the first 12 hours. Both the control subject and the patient showed a peak of phytanic acid radioactivity in the plasma at 6 hours. This disappeared rapidly in the normal subject and had virtually all disappeared by the second day, whereas the patient showed a second large peak of radioactivity at 2 days, and this fell only slowly over the next 4 days. The initial peak presumably reflects the presence of labeled phytanic acid in chylomicrons: studies in rats show that the *phytol* is partly converted to phytanic acid during the course of absorption, 15% of the label in the chylomicrons being present as phytanic acid and the remainder as *phytol*. The second large peak in radioactivity in the patient with Refsum's syndrome presumably reflects secretion of phytanic acid into the plasma lipoprotein compartment. The extremely slow subsequent disappearance and the very poor conversion to $C^{14}O_2$ strongly suggests that the metabolic error in this disease lies in the pathway for oxidation of the branched -chain structure of *phytol* and/or phytanic acid. This conclusion is supported by concurrent independent studies at Rikshospitalet showing that patients with Refsum's syndrome have a much lower capacity to

convert capric acid to its omega oxidation derivative, sebacic acid.

A screening program was initiated to determine whether phytanic acid or other abnormal fatty acids could be found in the plasma of patients with neurological diseases similar to Refsum's disease. Patient material from a number of medical centers around the country has been studied, as well as patient material at the Naval Medical Center and on the service of Dr. Engle and Dr. Carr here at N.I.H. More than 20 cases of retinitis pigmentosa have been examined, and a few patients with amyotrophic lateral sclerosis, Dejerine-Sottas syndrome, spino-cerebellar degeneration, multiple sclerosis, familial dysautonomia, Sjögren-Larsson syndrome, various ataxias and peripheral neuropathies have been examined. In none of these was phytanic acid or any other abnormal fatty acid found. From all of these results taken together, it appears that patients with Refsum's syndrome display a highly specific metabolic error; that is, the accumulation of phytanic acid is probably not secondary to the nerve degeneration. The isotope studies indicate that biosynthesis is not a major source, but that phytanic acid probably has an exogenous origin. *Phytol* is shown to be a very effective precursor, but it remains to be established whether the dietary intake of *phytol* itself or of other potential precursors is adequate to explain the accumulation in these patients. Whatever the origin of the phytanic acid, the present results indicate that the metabolic error lies in the oxidative pathway. It is quite possible that the enzyme defect embarrasses a metabolic pathway or pathways in addition to that for phytanic acid degradation. Studies are in progress to determine the steps involved in the breakdown of phytanic acid so that the specific site of the block in Refsum's syndrome can be pinpointed.

Studies of Lymphatic Lipids and their Origin

It has been known for some time that even in the fasting animal there is a continuing production of lipids appearing in the lymph drained from the thoracic duct. However, the origin and nature of this "endogenous lymph lipid" has not been previously determined. Us-

ing techniques for cannulation developed in this laboratory, this problem has been investigated in rats maintained on a fat-free diet. It was shown that most of the lipid appearing in the thoracic duct lymph actually has its origin in lymphatics draining the small intestine, i.e., it does *not* come primarily from liver lymph. The material is mostly contained in very low-density lipoproteins floating at density 1.006 in the centrifuge. Because the concentration in the lymph is higher than that in the plasma, it seems unlikely that this is derived from the plasma. Studies after injection of radioactive fatty acids show that the circulating FFA fraction is not a major source. Lipids delivered to the duodenum with bile may account for about half of the endogenous lymph lipids, and the remainder presumably synthesized directly in the intestinal wall.

The extent to which the intestine contributes lipids to the plasma has never been established with certainty. Recent studies in man suggest that this may be of significance. The present results in the rat, if combined with ancillary studies of plasma lipoprotein origins, may be of great importance in resolving this problem.

In conjunction with the studies of C¹⁴-phytol metabolism described above, an intensive study of its lymphatic absorption has been carried out. Although most of the phytol absorbed enters by way of the lymph in most animals studied, an occasional rat appears to absorb phytol to a significant degree by another route. This is currently under investigation. Most of the radioactivity in the lymph is phytol, but this is largely present in a combined form, possibly long-chain fatty acid esters of phytol. About 15% of the radioactivity is found in phytanic acid, indicating conversion of the alcohol to the acid in the intestinal wall. The phytanic acid also appears to be present largely in combined forms.

Studies of Membrane Lipoproteins

Little is known about the basic structure of membrane lipoproteins. These proteins are only sparingly soluble in ordinary media, and this has made difficult the fractionation and characterization of them. Some progress has been made on this important problem by sub-

jecting red cell ghosts to a fractionation procedure in which sodium desoxycholate is used to solubilize the membrane proteins. A fraction has been obtained which appears to be homogeneous by immunochemical criteria and which shows a single peak in the analytical ultracentrifuge. The molecular weight is in the neighborhood of 200,000 to 250,000. Attempts will be made to further characterize this fraction.

Further Studies of the Cholesterol Biosynthetic Pathway

Earlier studies using triparanol as a tool for inhibiting cholesterol synthesis suggested that $\Delta^{7,24}$ -cholestedienol was on the normal pathway for cholesterol biosynthesis. Radioactive $\Delta^{7,24}$ -cholestedienol was prepared synthetically and this has now been shown to be readily converted to cholesterol by homogenates of rat liver. In addition, it was shown that Δ^{24} -cholestolenol is convertible to cholesterol, although less readily than the dienol. These studies provide further evidence that $\Delta^{7,24}$ -cholestedienol is an intermediate in cholesterol synthesis, and they show further that the double bond at the 7 or at the 5 position is not an obligatory requirement for the side-chain reductase, i.e., the Δ^{24} -bond can be reduced even when the steroid nucleus is fully saturated.

Section of Molecular Disease

Protein Structure and Function

1. The parathyroid hormone has been sufficiently purified to begin analysis of its amino acid sequence. The immunochemical assay of the hormone in plasma has been developed to levels of sensitivity and reproducibility sufficient for study of clinical states of abnormal parathyroid function.

2. Studies of the covalent structure and conformation of ribonuclease and several of its smaller derivatives have opened up some further methodological approaches to evaluation of three dimensional conformation of enzymic proteins.

3. Collaborative studies have extended to two years the highly successful treatment of

the inborn-error of metabolism, cystinuria, by the use of penicillamine.

Plasma Lipids and Lipoproteins

Several major advances were made during the year toward clarifying the nature and functional relationships of the plasma lipoproteins and toward understanding clinical abnormalities in their concentrations.

1. The paper electrophoretic technique of Lees and Hatch was set up and thoroughly evaluated. It was concluded this method separates lipoproteins into the maximum number of groups useful for most clinical purposes at the present time. The identify of these four groups (alpha, beta, and pre-beta lipoproteins, and chylomicrons) was established and cross-correlated with other means of separating lipoprotein and "particles". For purposes of studying pre-beta lipoprotein (endogenous glyceride) conditions were determined for reproducible augmentation of the concentration of these lipoproteins (carbohydrate-induction) in normal subjects and patients.

An entirely new classification of familial hyperlipoproteinemia was then proposed. It is based on the lipoprotein patterns in plasma of patients. The conditions include a fasting sample and that the patient shall have been on a "normal diet". Five different patterns have been seen recurrently and correlate with other clinical features in such a way as to suggest that different genotypes are represented. They have been assigned simple designations—Types I - V. Use of this systematic approach has allowed us to segregate what appear to be more different diseases among this group of disorders than has ever been recognized. The system has already been adopted by a number of laboratories here and abroad. It will serve as a frame of reference for future revision and expansion.

2. As pre-beta lipoprotein levels were observed concomitant with those of alpha and beta lipoproteins it became apparent that pre-beta lipoprotein (very low density lipoproteins) might consist of beta lipoprotein plus alpha lipoprotein plus triglyceride. This was confirmed by a number of experimental approaches, including decomposition of pre-beta

lipoproteins and immunochemical identification of the products. It was then shown that subjects having no beta lipoprotein could neither be carbohydrate-induced nor make pre-beta lipoprotein. Patients deficient in alpha lipoprotein have an increase in plasma glycerides but cannot make pre-beta lipoprotein.

From this and other evidence a tentative functional view of plasma lipoproteins has been advanced that considers the problem of neutral fat transport in terms of three major variables, alpha and beta proteins (each having a certain complement of cholesterol and phospholipid) and triglyceride. This represents a simplification of lipoproteins to what might be called the irreducible minimum of components. This provides a structure within which other peptides (reported by other laboratories) and various subfractions as obtained by density differences, et cetera, can be examined in terms of specificity and functional essentiality. These views are being summarized in a major review.

Section on Chemistry

The Section on Chemistry is pursuing three main lines of activity. 1. The structural analysis, synthesis and biosynthesis of new compounds of biological origin. 2. Development of methods of analysis for compounds of biological importance. 3. Rendering assistance via our organic chemical approach to biological and medically oriented groups throughout the Institutes. Purchase of a new mass spectrometer has accentuated the second and third items during the recent year as we have attempted to educate other groups to its inherent analytical utility.

Specifically, during the past year, individuals in this section, either alone or in collaboration with others, have:

1. Synthesized labeled intermediates related to phytol for a study of Refsum's disease.
2. Identified acids resulting from the radiolysis of glycine.
3. Investigated the analysis of castor oil.
4. Identified an isomer of elaidic acid.
5. Proved the structure of a new estrogen, = 11β -estetrol.

6. Developed a method of analysis of estrogenic ketones in urine.
7. Confirmed the structure of a new analgesic of the benzomorphinan series.
8. Assisted in the structure proof of an insect sex attractant.
9. Developed and proved the biosynthetic pathway of formation of the methylene-bis-phloroglucinols.
10. Revised the structure of a series of alkaloid degradation products.
11. Assigned structures to two new isoflavones, milletine A and B.
12. Developed methods for the characterization of fern phenols.
13. Synthesized a structural isomer of the alkaloids mesembrine and crinine.
14. Initiated a mass spectral study of sesquiterpene lactones.
15. Introduced evidence indicating the need for caution in interpreting mass spectra via the element map technique.
16. Developed and proved the structure of several new alkaloids of *Astrocasia phyllanthoides*.
17. Developed methods for the future pu-spectra via the element map technique. rification of components of the kallikrein systems from human urine pancreas and blood.
18. Proved the location of deuterium in-troduction into demosterol.

In the future, it is assumed that increasing emphasis will be placed on biosynthesis and natural product structural analysis employing the techniques of n.m.r. and mass spectroscopy.

LABORATORY OF CLINICAL BIOCHEMISTRY

Amine Biogenesis and Metabolism

Tyrosine hydroxylase has been purified 400 to 500 fold from beef adrenal medulla. The purified enzyme shows a definite requirement Fe^{++} and reduced tetrahydropteridines which is apparently a characteristic of many other aromatic hydroxylases. The same enzyme can also convert *L*-phenylalanine to tyrosine and the latter to DOPA. The ability of the one enzyme to catalyze two consecutive hydroxylation

steps offers a means of studying the mechanism of hydroxylation.

It appears fairly certain that tyrosine hydroxylase is the rate-limiting step in the over-all production of norepinephrine in the sympathetic nervous system. It has been proven that compounds such as α -methyl-tyrosine and for 3-iodotyrosine lower tissue norepinephrine levels by virtue of inhibiting the initial rate-limiting step. In collaborative studies with the Experimental Therapeutics Branch, it has been shown that administration of α -methyl-tyrosine diminishes the excretion of norepinephrine metabolites in the urine of patients. It would appear, therefore, that inhibition of tyrosine hydroxylase will make it possible to lower production of norepinephrine in man. This has never before been accomplished, by chemical means.

Being the rate-limiting step in the over-all process, tyrosine hydroxylase may be susceptible to other regulatory influences. Some evidence suggesting changes in norepinephrine synthesis in response to sympathetic stimulation has been obtained. It would also appear that the three enzymes involve in norepinephrine formation, tyrosine hydroxylase, dopa-carboxylase and dopamine- β -oxidase, occur in the same subcellular fraction and comprise a norepinephrine synthesizing (and storage) organelle.

Collagen and Hydroxyproline

The cell-free system (chick embryo) for conversion of proline-C¹⁴ to collagen hydroxyproline has provided additional information as the site of proline hydroxylation. First, by incubating microsomes with proline-C¹⁴, for short periods of time under nitrogen, it is possible to incorporate proline-C¹⁴ and obtain very little protein-bound hydroxyproline-C¹⁴. Such "proline-prelabeled" microsomes can be washed free of diffusible radioactivity and, on subsequent incubation in the presence of ascorbic acid, another heat stable cofactor and oxygen, as much as 10 percent of the protein-bound proline is converted to hydroxyproline. Furthermore, the "proline-prelabeled" microsomes can be treated with a highly specific bacterial collagenase to yield peptides which con-

tain proline-C¹⁴ and little hydroxyproline-C¹⁴. All these observations are consistent with the mechanism previously proposed by this laboratory; proline is activated and incorporated into protein (collagen precursor) and is subsequently hydroxylated in peptide linkage. Evidence for a hydroxyproline deficient collagen precursor has now been obtained from many tissues other than the chick embryo.

In collaboration with Dr. Bernhard Witkop, it has been possible to show that the hydroxylation of proline involves a selective displacement of the hydrogen in the 4-trans position of the proline ring by the entering hydroxyl group. This information was obtained with synthetically prepared cis and trans 4-tritioprolines. It was also possible to prepare cis and trans 4-fluoroprolines and show that both are incorporated into proteins. Of most interest was the finding that trans fluoroproline is oxidatively defluorinated and converted to collagen.

The circulating "collagen" previously described by us has been purified about 100 fold. Studies in the Experimental Therapeutics Branch show that this protein rises in patients with certain tumors and may be an important diagnostic tool.

Amino Acid Uptake by Animal Tissues

It has been possible to obtain several synthetic amino acids which fluoresce in the visible region. These compounds, amino-naphthalanines, are taken up by ascites cells in a manner comparable to other amino acids. The studies are designed to investigate the physical state of the fluorescent amino acid within the cell. If it is attached to a "carrier" this should show up by changes in several parameters such as quantum yield, excitation maximum, fluorescence maximum and rate of depolarization.

Biosynthesis of Nitrogen Containing Lipids

Procedures for assay of palmitoyl-ethanolamide have shown that the compound is present in highest concentration in brain and liver. When labeled palmitic acid, serine or ethanolamine were administered to animals, the isolated palmitoyl ethanolamide was found to be labeled. The enzymatic formation of the fatty acid amide has presented an enigma since en-

ergy sources are not required. However, more recent studies suggest that the fatty acids first combine with protein in some manner and that amines such as ethanolamine, tyramine, phenethylamine are transferred to the fatty acid by displacing a protein. These findings may be of great significance with respect to fatty acid metabolism and transport.

RNA Metabolism in Brain

It was observed that the uptake of labeled purines, pyrimidines and their nucleosides into brain slices varied with age and with the portion of the brain utilized. Further studies have shown that some nucleosides and purine metabolizing enzymes are asymmetrically distributed in the brain and that some of these enzymes undergo marked increases following birth and during growth. The significance of these changes and the factors which regulate them are under investigation.

Proteins and Peptides

With the reagent, dimethyl amino naphtalene sulfonyl chloride (DANSYL), it has been possible to convert vasoactive peptides to their corresponding DANSYL derivatives which are highly fluorescent. With the help of this procedure, it has been possible to isolate and characterize the major vasoactive peptide from wasp venom. The amino acid composition has been determined and it has been shown that on treatment with trypsin the peptide is cleaved to yield a smaller vasoactive peptide which has been identified as glycyl bradykinin. Studies on the over-all structure of the mother peptide are being continued. It is of interest that the bradykinin peptide sequence exists in the wasp.

The presence of β -aspartyl residues in collagen has been verified. β -Aspartyl peptides have also been found in fibrin and evidence has been accumulated to indicate that β -aspartyl linkages are increased in the conversion of soluble to insoluble fibrin. Evidence has also been obtained which indicates that the lathyritic agent, β -aminopropionitrile, reacts with β -aspartyl crosslinkages in both collagen and fibrin.

It has been established that the formation of the peptide antibiotic actinomycin involves mechanisms distinct from the known mechanisms for protein synthesis. Using D-valine as an inhibitor of actinomycin synthesis, evidence has been obtained for the accumulation of precursors of the phenoxazinone ring. 4-Methyl-3-hydroxyanthranilic acid has been detected as an intermediate in actinomycin synthesis and appears to accumulate during D-valine inhibition. It appears that D-valine either prevents the synthesis or attachment of the pentapeptide chains to the phenoxazinone chromophore. It has been shown that the bulk of added D-valine-C¹⁴ which remains in the medium after incubation occurs as N-succinyl D-valine, the balance as D-valine peptides.

Aromatic Hydroxylation and Metabolism

The L-phenylalanine hydroxylase from *Pseudomonas sp.* has been purified to the extent of showing an absolute requirement for Fe⁺⁺ and tetrahydropteridines. Bacterial extracts have been shown to contain a factor which can replace synthetic tetrahydropteridines as a cofactor. On further investigation, it was shown that the organism can make a hydroxylation cofactor from guanosine triphosphate. This conversion is being investigated. The bacterial cofactor appears not to be exactly the same as the cofactor for liver phenylalanine hydroxylase.

Studies were also carried out on some of the enzymes involved in the *de novo* biosynthesis of aromatic amino acids in *Pseudomonas sp.* The enzyme, prephenate aromatase, was purified and its properties determined. It was also shown that addition of phenylalanine to the medium not only induces phenylalanine hydroxylase, but also inhibits prephenate aromatase; added tyrosine stimulates the aromatase. All these findings suggest that there exist regulatory mechanisms for turning off aromatic amino acid formation in *Pseudomonas sp.* when phenylalanine is present in the medium.

5-Tritium labeled tryptophan has been prepared and is being investigated as a substrate for tryptophan hydroxylase. If it is successful, it should provide a rapid, simple and sensitive procedure for measuring this enzyme.

Vitamin B₁₂ and Methionine

Additional evidence has been obtained that reduction and alkylation are essential features of the cobamide function in methionine biosynthesis. Alkylation of the cobamide has been achieved by chemical means; the requirements for chemical alkylation are identical to those for enzymatic alkylation with N₅-methyl-folate in that both require a reducing system and S-adenosylmethionine.

Studies have continued on the control of one-carbon metabolism in *E. coli*. It has been found that the metabolism of N₅M₁₀-methylene tetrahydrofolate is affected by methionine and purines such as guanine. The former represses conversion of the methylene folate to the methyl derivative, the latter represses the conversion of methylene folate to the methenyl folate. These represent mechanisms by which the cell regulates methionine and purine biosynthesis.

Section of Biochemical Genetics

Although the base composition of RNA codons and many properties of the genetic code were clarified with the use of synthetic polynucleotides, the sequence of the bases within each codon remained unknown. During the last year we found a general method of great simplicity for determining the base sequence of codons and for investigating many aspects of codon recognition. The method depends upon the ability of trinucleoside diphosphates to direct the binding of C¹⁴-aminoacyl-sRNA to ribosomes prior to peptide bond synthesis. Thus, codon recognition was studied with trinucleotide templates. Since tri- but not dinucleotides served as templates in this system, a triplet code was demonstrated directly.

Since 5'-terminal, 3'-terminal, and internal codewords differ in chemical structure, three corresponding classes of codewords were proposed. The recognition of each class in this system was studied. The template efficiency of trinucleotide codewords was found to be modified greatly by terminal phosphate. Triplets with 5'-terminal phosphate were more active as templates than triplets without terminal phosphate. Triplets with 3'—or 3'(2')-terminal phosphate were markedly less active as tem-

plates. The modification of RNA and DNA codewords, converting sense into missense or nonsense codewords, was suggested as a possible regulatory mechanism in protein synthesis.

Enzymatic methods were devised by M. Bernfield and by P. Leder, M. Singer and R. Brimacombe to synthesize trinucleotides of known sequence, for two-thirds of the 64 possible trinucleotides are new compounds, not previously synthesized or isolated from RNA digests. Thus far we have prepared 45 trinucleotides and investigated their template activity for C¹⁴-aminoacyl-sRNA in this system. Nucleotide sequences of RNA codons for every amino acid have been demonstrated and functions have been ascribed to almost every triplet.

Recent results have permitted several generalizations to be made concerning the nature of the code. (A) Three classes of synonym codon sets have been found: 1) synonym codon pairs such as XpYpU and XpYpC, 2) synonym pairs such as XpYpA and XpYpG and 3) synonym codon sets such as XpYpU, XpYpC, XpYpA and XpYpG. Recognition of the 3'-terminal base clearly is most variable. In one or two cases, U = C at the 5'-terminal position. (B) In most cases the apparent template activity of one member of a synonym codon set differs from that of another. (C) Amino acids, which are structurally or metabolically related, often synthesized *in vivo* from a common precursor, have strikingly similar RNA codons. Such relationships would appear to reflect either the evolution of the code or direct interactions between amino acids and bases in codons. (D) Partial recognition of a triplet or alternate recognitions of bases possible may suffice during protein synthesis.

The capacity of trinucleotides to direct the binding of sRNA to ribosomes and the ease with which the process can be assayed provide a method for studying the base sequence and genetic function of each triplet codon. In addition, this method permits the detailed study of interactions between codons, sRNA, and ribosomes during the codon recognition process, and may provide opportunities to investigate mechanisms which control the rate of protein synthesis.

CLINICAL ENDOCRINOLOGY BRANCH

The activities of the Clinical Endocrinology Branch have involved studies on the adrenal cortex, studies of renal function with special reference to sodium, studies of calcium and phosphorus metabolism and miscellaneous other studies.

Adrenal Cortical Steroids

Patients with hypertension were studied to determine the presence or absence of aldosteronism. Aside from the direct measurement of excretion of the indirect measurement of secretion of aldosterone, the production of hypokalemia by means of a high sodium intake continues to be the best clinical index for aldosteronism. In each patient with aldosteronism a number of variables were evaluated in the attempt to gain insight into the mechanism of aldosteronism resulting from adrenocortical hyperplasia as opposed to that resulting from adrenocortical tumor. Plasma renin was measured in all cases and in addition, extensive studies of renal hemodynamics with special reference to difference between the kidneys, and measurements of blood volume and its change with sodium intake were performed. Each patient with aldosteronism is eventually subjected to exploration. Whereas no criteria presently available clearly distinguish the two types of aldosteronism, the search continues for physiologic maneuvers which may suppress the aldosteronism of hyperplasia. Suppression of aldosterone secretion by the use of 11-hydroxylase inhibitors together with ACTH inhibitors has been carried out for periods up to 30 days without demonstrable effect on blood pressure in patients with aldosteronism of either cause.

Studies on the metabolic pathways for biosynthesis of adrenal cortical steroids in the adrenogenital syndrome suggest that the conventional explanation for the difference between salt-losing and non-salt-losing adrenogenital syndrome (simple difference in "degree" of inhibition of 21-hydroxylase) is inadequate. Patients with the non-salt-losing syndrome were found to secrete quantities of aldosterone far greater than normal and to increase this secretion abnormally with physiologic stimuli

such as sodium deprivation. This has been interpreted to indicate adequate (and indeed excessive) 21-hydroxylation of progesterone in such patients, the sole enzymatic defect appearing to be a failure of 21-hydroxylation of 17-hydroxyprogesterone. Presumably, patients with salt-losing adrenogenital syndrome have a block in 21-hydroxylation of progesterone as well. Studies of the control of steroidogenesis by the adrenal cortex include a direct comparison *in vivo* and *in vitro* of the effect of sodium deprivation with that of angiotensin. Unless sodium deprivation in fact increases adrenal cortical steroid secretion via the hypersecretion of renin and of angiotensin, it should be possible to distinguish the sites of the effects of these two agents in biogenetic pathways for aldosterone. Results suggest that the mechanisms of actions of the two agents are indeed different, as the addition of a precursor "below" pregnenolone could inhibit aldosterone production in the presence of angiotensin whereas it could stimulate aldosterone production in the adrenal cortex under the stimulus of salt deprivation. According to current concepts, this strongly suggests that sodium deprivation has an action beyond the site of incorporation of $\Delta 5$ pregnenolone into the biogenetic pathway for aldosterone.

In pursuit of the observations that insufficiency of carbohydrate-active steroids produces marked increases in sensitivity of taste and smell and moderate elevations in the rate of nerve conduction velocity, studies were instituted in cats before and after adrenalectomy. The increase of nerve conduction velocity following adrenalectomy was confirmed. Preliminary results with extraction of nerve and brain tissue before and after incubation suggest that cortisol, corticosterone and aldosterone are contained in nerve tissue and may be released from nerve tissue during incubation. The steroid content of nerve tissue did not appear to be significantly decreased after adrenalectomy despite clear evidence of adrenal insufficiency. Since patients with the adrenogenital syndrome were found also to exhibit the increase in sensitivity for taste and smell characteristic of patients with adrenal cortical hypo-function specifically lacking carbohydrate-active steroids, these thresholds as well as that of

hearing were measured in such patients before and after administration of carbohydrate-active steroids. The patients showed increased sensitivity of all thresholds measured; they could detect sound frequencies both below and above the normal range. Whereas carbohydrate-active steroids returned their taste and smell sensitivity to normal (albeit at a rate significantly lower than that for classical Addison's disease), it had no effect on the hearing sensitivity.

The relationship of hormones to the function of nerve cells was further pursued by tissue culture procedures in which glial cells and nerve cells could be studied separately. Preliminary studies indicate that carbohydrate-active steroids may increase the cloning efficiency of cultured HeLa cells; effect on nerve cells is under investigation. In further pursuit of the relationship between taste and smell sensitivity and hormonal function, detection and recognition thresholds for taste and smell were measured in a wide variety of clinical syndromes. Temporary adrenal insufficiency medicamentosa produced with metapirone which inhibits 11-hydroxylation of adrenocortical steroids, was associated with immediate increases in sensitivity for taste and smell. This appeared to be a drug effect as it was not prevented by the simultaneous administration of hydrocortisone. Studies on patients with hypertension confirmed previous evidence from this laboratory that taste and smell for sodium and other modalities are normal in hypertension and exhibit normal circadian variability. Study of smell sensitivity was pursued in a wide variety of patients without known abnormality of adrenocortical function. It was found that anosmia and hyposmia do occur in six syndromes which can be distinguished from each other. In the first there is true anosmia and there may be hypogonadism but the disorder is not familial. In the second there is marked hyposmia which is familial. In the third there is moderate hyposmia which is familial and there is also hypogonadotropic hypogonadism. The fourth syndrome is associated with surgical removal of primary and secondary centers for taste and smell, the fifth with anatomical abnormalities of the mid-line including cleft palate and other oral defects and the sixth includes the malab-

sorption syndrome in which there is hypovitaminosis A. In none of these disorders has adrenocortical dysfunction been established.

The gross deficiency in taste sensitivity of patients with familial dysautonomia was found to depend in part upon absence of circumvalate and fungiform papillae in which the taste buds are found; it was further found that parasympathomimetic agents could return taste sensitivity for a short period. This indicates that some ability to taste is present without specific taste buds.

Studies of Renal Control of Salt and Water Metabolism

We have shown that suppression of adrenergic activity with blocking agents such as guanethidine promotes sodium loss during sodium deprivation and limits the sodium retention resulting from the use of sodium-retaining steroids. The results thus suggest a normal role for catecholamines in the renal control of sodium excretion. This role was further examined in patients with various types of disorder involving formation of edema and in dogs in which pathological retention of sodium and water was induced surgically and pharmacologically. In addition, the effects of bradykinin were tested and compared with those of agents known to affect renal sodium excretion. In patients with a tendency to edema formation, beta adrenergic blockade with propanolol induced no further exacerbation of sodium retention, nor did the beta adrenergic blockade appear to decrease the sodium retention resulting from the use of sodium-retaining steroids. Alpha adrenergic blockade with dibenzylene on the other hand, as well as blockade of both alpha and beta receptors with guanethidine decreased the sodium retention induced by sodium-retaining steroids with two exceptions: In both of the exceptions sodium retention was aggravated by blockade; this finding led to further studies of cardiac function which was found deficient in both patients. The results were taken to indicate that the dependence of the myocardium on catechol activity, unmasked by adrenergic blockade, induced sodium retention despite blockade of renal alpha receptors as well.

Dogs were treated with constriction of the superior vena cava and given sodium-retaining steroids to induce rapid formation of ascites. Sodium diuresis was induced in such dogs with ganglionic blockade produced with pentolinium tartrate; in a number of instances, there was a decrease of mean arterial pressure and a decrease in the glomerular filtration rate, findings which indicate clearly a role for the adrenergic nervous system in tubular reabsorption of sodium. Bradykinin could also increase sodium excretion despite decreases in glomerular filtration rate in normal subjects; the sodium loss could be prevented by adrenergic blockade. The results suggest a complex role for bradykinin which may involve release of angiotensin or of renin.

The relationship of hormonal and nervous activity to renal control of sodium excretion could not be clarified without more intimate knowledge of renal circulation and studies were instituted to measure cortical and medullary flow separately. With the use of Xenon¹³³ injected into the renal artery, exponential decay curves were derived which, upon compartmental analysis, could provide indices of blood flow in various parts of the renal circulation. The application of this method to the variables under study is in progress.

The special natriuresis which accompanies fasting was further studied by metabolic balance in human subjects. The natriuresis could not be attributed to Ketone body formation alone, although it appeared likely that these studies contributed in supplying anions requiring excretion. Results suggest that the natriuresis results in part from a failure of hypersecretion of aldosterone which normally results from a decrease in sodium intake. It appears further that potassium depletion may contribute to this failure of aldosterone secretion. Finally, it appears that carbohydrate alone can restore the ability to secrete aldosterone normally. The mechanism for this function of carbohydrate is under study.

Studies of the salt and water excretion in the syndromes of inappropriate secretion of anti-diuretic hormone were continued in a 5-year old child with a severe gunshot wound in the region of the hypothalamus. Inappropriate secretion of antidiuretic hormone was document-

ed and shown to disappear with the passage of time. As the patient had developed a marked resistance to the stimulus of metapirone to secretion of ACTH, it was suggested that a deficiency of cortisol may play a part in the syndrome. If the inappropriate secretion of anti-diuretic hormone results from the hypothalamic lesion, it is possible that other patients with the syndrome may demonstrate an inability to respond to metapirone.

In pursuit of the relationship between sodium excretion and adrenergic activity, extensive preliminary studies of epinephrine and norepinephrine excretion have been carried out in patients with a wide variety of diseases. The normal range for excretion of free and bound epinephrine and norepinephrine has been established; data for all age groups can be fitted to a single scale when "corrected" for surface area. Normal excretion of free and bound epinephrine and norepinephrine was shown to persist under the influence of ACTH and in patients with congenital adrenal hyperplasia, panhypopituitarism, acromegaly, hypoparathyroidism, hyperthyroidism, pseudohypoparathyroidism and idiopathic edema; normal values for free and bound epinephrine and norepinephrine were found in one patient with sub-total and one with total adrenalectomy, indicating a non-adrenal source for urinary epinephrine. Bound epinephrine was found to be very low in urine of patients with familial dysautonomia. Bound norepinephrine was the single consistent abnormality of the epinephrine-norepinephrine excretion of the patients with pheochromocytoma. The relationship of catecholamine excretion to the state of sodium balance is under investigation.

Studies of Calcium and Phosphorus Metabolism

A large number of studies of the distribution of calcium⁴⁷ have been carried out with concomitant observations of the specific activity of blood, urine and stools in an attempt to construct a model which will explain the events in metabolic bone diseases and in patients with various diseases with and without treatment such as sarcoidosis with and without vitamin D, osteoporosis with and without estrogen, and in normal subjects to measure the effect of cal-

cium loading, of phosphate deprivation, and of growth hormone. Studies of conventional dynamics show marked acceleration of bone turnover in patients with sarcoidosis, the change having the same characteristics as that induced by vitamin D in normal subjects. This finding suggests that the bones, like the gastrointestinal tract in sarcoidosis, may suffer hypersensitivity to vitamin D. Calcium infusion markedly elevated calcium turnover in normal subjects, a finding that suggests a simple mass action at bone surfaces and one which indicates the need for considerable caution in the interpretation of conventional dynamic data. Simple hypercalcemia may introduce spurious variation.

Phosphate deprivation was found to alter bone dynamics profoundly in normal subjects, inducing extremely rapid bone turnover, increases in serum calcium and marked increases in urinary calcium. Indeed, it was found that in hypoparathyroidism phosphate deprivation alone could restore serum calcium to normal over long periods and increase urinary calcium to super-normal quantities over the same period. The dynamic data indicate further that phosphate deprivation induced by Maalox-amphogel which lowers urinary phosphorus to near-zero values increases the absorption of calcium in the gastrointestinal tract. At the same time, it increases skeletal resorption of calcium.

The use of urinary hydroxyproline as an index of collagen turnover has proved of great value in assessing metabolic bone disease. An unexpected finding developed with the experimental elevation and depression of serum calcium in hyperparathyroidism. It had been expected that this would not alter hydroxyproline excretion if parathyroid hormone secretion was not changed. Consistently, the opposite was true. A survey of patients to define the intrinsic gastrointestinal ability to absorb calcium was continued. The majority of normal subjects show little change in calcium absorption and in urinary calcium as dietary calcium is increased twenty-fold. Rare normal subjects and a number of subjects with a tendency to stone formation show linear increase in urinary calcium with increases in dietary calcium. It appears likely that the two groups are genetically determined.

Studies designed to clarify that hypophosphatemia of rickets and the effect of vitamin D in restoring it have been continued with clinical studies in hypophosphatemic rickets and with experimentally-produced rickets in puppies. In the latter, it was clearly shown that the hypophosphatemia of rickets depends on secondary stimulation of parathyroid activity which results from the hypocalcemia produced by deficiency of vitamin D. In the rachitic puppies in which the abnormalities of phosphorus metabolism (decrease of serum phosphate and increase of phosphate clearance) quickly returned towards normal after parathyroidectomy, exogenous parathyroid extract has its usual effects, a finding which negates early reports that parathyroid hormone requires vitamin D for its effects. Hydroxyproline excretion was found to be elevated in the rachitic puppy, a finding in support of the view that urinary hydroxyproline results in part at least from release of soluble pre-collagen associated with bone formation and not exclusively from the release of collagen as bone is destroyed. There is no evidence of bone destruction in the rachitic bones of these animals.

Miscellaneous Studies

The development of atherosclerosis was studied by measurement of the rate of accumulation of I^{131} labeled lipoprotein in the aorta of rabbits before and after the production of atherosclerosis and hyperlipidemia. Development of atherosclerosis was associated with marked increase in accumulation of lipoprotein suggesting that such increase of permeability may play a part in the development of atherosclerosis.

Numerous studies of circadian rhythm with especial reference to variables associated with the adrenal cortical function have been carried out in normal subjects and some in patients suspected of diseases of desynchronization such as those with periodic disorders. Transverse maps of adrenal cortical variables were carried out in Australia to verify the essential similarity of the external and internal timing of adrenal events at 145° east longitude to those in the United States. In a patient with periodic fever and hypertension, extensive studies of

hemodynamic and adrenal events suggested that different periods may appear for grip strength, blood pressure, pulse and respiration as opposed to time estimation. Such a difference could be a first indication of a disease of desynchronization.

In pursuit of other indices for the measurement of periodic hyperpyrexia, the branch has developed and tested extensively a new method for the measurement of serum etiocholanolone, the first of the five-beta steroids implicated in the disorder of periodic hyperpyrexia. Elevated values were indeed found in a patient with carcinoma of the rectum but normal values have been found in a number of patients with periodic fever; thus far, no confirmation of the syndrome of high fever resulting from excess of plasma etiocholanolone has been obtained.

Effect of penicillamine in cystinuria was studied by clinical and biochemical means. It was shown that penicillamine decreased effectively and consistently the urinary cystine in patients with cystinuria; surprisingly, it produced also a decrease in total half-cystine, that bound to penicillamine being much less than the amounts originally present in the urine. In extensive clinical trials of the effect of penicillamine it was found possible to control stone formation effectively and to institute the dissolution *in vivo* of stones in four subjects.

Studies of the abnormalities of renal function in cystinosis were continued. The effect of penicillamine in decreasing the basic storage of cystine is under study. It has been found that patients with cystinosis show also a poor response to metapirone, a finding suggesting additional disorder of pituitary function, possibly related to the growth defect characteristic of this syndrome.

A disorder in infants characterized by hypercalcemia but differing from the classical syndrome of "idiopathic hypercalcemia of infants" in the association of hyperlipidemia and hypercalcemia has been studied in two subjects. Hyperlipidemia was shown to be dependent on dietary carbohydrate. It was found that hypercalcemia was not directly dependent upon the elevation of blood lipids. The elevation of blood lipids may reflect the abnormality in vitamin D metabolism which in turn may be related to the hypercalcemia.

The finding that the serum and dialysis fluid of patients with uremia may contain "toxic" substances was pursued. A previous observation that HeLa cells are killed by the eluates of such fluid after passage through Sephadex was confirmed. The toxic material appeared in two separate peaks during the elution. The uptake of glucose by normal red cells was effectively blocked by the peaks which have a toxic effect on HeLa cells and could kill normal mice. Continued studies are designed to identify the toxic substances. An unexpected finding developed during prolonged peritoneal dialysis of a patient with vitamin D intoxication in the demonstration that over a million units of vitamin D were removed directly by dialysis.

EXPERIMENTAL THERAPEUTICS BRANCH

A variety of investigations chiefly of immediate or at least potential clinical significance have been continued. Findings obtained will be considered under the following headings: 1) Biochemistry and Pharmacology of Vasoactive Amines, 2) Studies on the Kinin Peptides, 3) Chemistry of Selected Proteins and 4) Miscellaneous.

Biochemistry and Pharmacology of Vasoactive Amines

It was shown previously that single doses of two inhibitors of histidine decarboxylase(s), 4-bromo-3-hydroxybenzyloxyamine (NSD 1055) and the α -hydrazino analogue of histidine (MK 785), produced marked decreases in urinary and tissue histamine levels in female rats, after intraperitoneal injection. In subsequent studies it was found that only slight decreases in histamine levels in guinea pig tissues and urine occurred even after repeated administration of these compounds. Further, no changes were observed in rats following oral dosing. Since leaving the department July 1, 1964, Dr. R. J. Levine has shown an inhibitory effect of these agents on gastric secretion in the rat. We have observed no changes in gastric secretion or urinary histamine in human subjects, however, following short term administration of NSD 1055 orally. Additional studies are planned with both agents in patients.

The first step in the biosynthesis of serotonin

is the hydroxylation of tryptophan to form 5-hydroxytryptophan. A tryptophan hydroxylase has been partially purified from neoplastic mouse mast cells and characterized. The enzyme is a typical aromatic ring hydroxylase and has an absolute requirement for oxygen, with a tetrahydropteridine as a specific reducing source. It is unusual in showing a nearly complete and specific dependence on ferrous iron. Although phenylalanine is also a substrate, the enzyme can be distinguished from the phenylalanine hydroxylase of rat liver. It has the lowest K_m for tryptophan (4×10^{-5} M) of any tryptophan hydroxylating system studied to date. Current emphasis is on discovery of inhibitors of the enzyme, as a possible approach to selective blockade of serotonin biosynthesis *in vivo*.

The hydroxylation of tyrosine to dopa has been shown previously to be the rate-limiting step in the biosynthesis of norepinephrine. Several compounds found by scientists in LCB to block tyrosine hydroxylase *in vitro* have now been studied in animals, and preliminary observations with two of the compounds have been made in man. The first and most potent compound studied was α -methyl-p-tyrosine (α MPT). Repeated dosage of guinea pigs produces almost 100% depletion of tissue norepinephrine without affecting serotonin levels. Numerous experiments indicate the mechanism of depletion to be by blockade of synthesis though observations with radioactive α MPT indicate that there is some conversion to α -methyl-catecholamines, raising the possibility of additional types of action of the compound during its prolonged administration. Three other tyrosine hydroxylase inhibitors, 3-iodo-tyrosine, 3-iodo- α -methyl-tyrosine and α -methyl-phenylalanine, have also been found to reduce endogenous levels of catecholamines but not as effectively as with α -MPT. The pharmacologic effects observed with α -MPT and to a lesser extent with the other inhibitors in cats, rats and guinea pigs include: reduced motor activity, sedation, slight meiosis, reduced tyramine pressor responses and reduced responses of the nicotitating membrane (cat) to nerve stimulation. Interestingly, no lowering of blood pressure has been observed. In a single patient with pheochromocytoma, e-iodo-tyrosine in maxi-

mum dosage of 2.0 gm/24 hrs. was without detectable chemical or pharmacologic effect. Alpha-MPT has been given in maximum dosage of 1.2 gm/24 hr. to several patients with essential hypertension and also three patients with pheochromocytoma; effects observed include marked drowsiness which tends to wane with continued treatment, persistent tranquilization in some patients, significant decrease in urinary excretion of vanilmandelic acid (VMA) and lowering of blood pressure in the patients with pheochromocytoma but not definitely in those with essential hypertension. A broad clinical investigation of tyrosine hydroxylase inhibitors in man is contemplated.

In an attempt to study the turnover rate of endogenous norepinephrine in man, H^3 -dopa has been administered to two subjects and the specific activity of urinary dopamine and norepinephrine studied as a function of time. While both amines were highly labeled, the specific activity of urinary dopamine failed to decrease below that of norepinephrine, negating calculations of turnover rate for the latter amine. The feasibility of calculations of dopamine turnover rate are under investigation and the use of highly labeled tyrosine is planned.

Our tyramine pressor test for pheochromocytoma is now undergoing broad clinical testing under commercial sponsorship. In personal studies on about 200 patients with essential hypertension, "negative" tests have been observed. The same was true in five patients with renovascular hypertension. Positive tests, i.e., 20 mm. Hg. systolic blood pressure rise following 1.0 mg (or less) of tyramine intravenously, have now been obtained in 12 patients with pheochromocytoma. No definite false negative tests have occurred. Thus, our initial favorable impressions have been sustained for an additional year.

We have been prompted to study in more detail the role of diet and intestinal bacteria on the excretion of urinary amines. A report in the literature implies that elevated levels of urinary tryptamine and tyramine during treatment with a monoamine oxidase (MAO) inhibitor may not reflect changes in the metabolism of amines produced in tissues. By use of glucose diets and oral antibiotics it can be shown that the elevated amounts of tyramine and

tryptamine in urine during MAO inhibition are primarily of tissue rather than intestinal (luminal) origin.

Kinin Peptides

Previous studies have strongly implicated the kallikrein system in the pathogenesis of flushing in carcinoid patients. While amounts of bradykinin capable of producing a flush can be measured in hepatic vein blood of such patients using routine bioassay techniques, no current method is sufficiently sensitive and specific to measure the low levels of bradykinin in peptides adsorbed on a CM-Sephadex column. We have been exploring the preparation of fluorescent derivatives and separations by thin layer chromatography as the basis for chemical methods of measuring vasoactive peptides. Basic peptides are adsorbed on a CM-Sephadex column, eluted and fluorescent derivatives are produced which are then developed on thin layer chromatography and quantified in a fluorometer. It is thought that this chemical approach may yield sensitive and specific means of assay of bradykinin, as well as other basic peptides which may be found in biological materials. Further exploration of alterations of kinin peptides in health and disease is being deferred pending the development of improved methodology.

Chemistry of Selected Proteins

Measurement of the excretion of hydroxyproline peptides as an index of endogenous collagen metabolism has been continued in a number of disease states. In confirmation of previous work, elevations have been observed in thyrotoxicosis, Paget's disease, and in acromegaly wherein diagnostic usefulness is apparent. Levels of a hydroxyproline-containing protein in plasma, which we have termed "hypro-protein", have been determined in 101 normal subjects and in 146 patients with a variety of diseases. Normal levels range from 5 to 10 $\mu\text{g}/\text{ml}$ (expressed as hydroxyproline) with little age variation. Elevated levels up to 30 $\mu\text{g}/\text{ml}$ occur in a variety of clinical disorders, the most consistent and highest elevations being in Hodgkin's disease, Sjogren's syndrome, and in febrile-inflammatory states. Practical

use has been made of plasma hypro-protein assays in evaluating the "activity" of Hodgkin's disease, the levels returning to normal when patients are in remission. Studies on artificial fever (etiocholanolone and endotoxin) indicate that fever *per se* is not accompanied by elevations in hypro-protein. Studies are in progress to purify this protein and establish whether it is identical with known macromolecular precursors of the collagen fibril.

Preparatory to the investigation of collagen pools in man, the conversion of radioactive proline to peptide-bound hydroxyproline in tissues and urine has been studied in animals. Technical difficulties were encountered which we believe put into question the validity of many reported studies. Modifications in methodology are being perfected to assure that only radioactivity present in hydroxyproline will be measured. In another pre-clinical study, experimental lathyrism produced by injection of β -amino-propionitrile (BAPN) has been studied as a model of collagen dysfunction. Chemical changes include an increase in α -chain subunits in skin collagen and elevated excretion of urinary hydroxyproline. A protocol for pilot studies using BAPN in humans afflicted with diseases characterized by excessive collagen formation is being explored.

The ferredoxins are small, non-heme, iron-containing proteins which serve as electron carriers in low redox potential reactions in certain bacteria. The chemical characteristics of several clostridial ferredoxins have been examined. Ferredoxin from *Clostridium pasteurianum* has been examined in most detail; it is composed of a single polypeptide chain of 55 amino acids to which are bonded 7 molecules of iron and 7 molecules of inorganic sulfide. The amino acid sequence has been largely determined. Experiments in progress indicate that ferredoxin is a two-electron rather than a one-electron carrier as has been supposed. During the studies on ferredoxin, a red protein was crystallized from extracts of *C. pasteurianum*. Its electron carrying properties and molecular weight were found to be similar to those of ferredoxin though it has a lower content of iron, lacks inorganic sulfide, and has a somewhat different amino acid composition. It is of great interest to us that non-heme, iron-con-

taining peptides have recently been described in mammalian systems; explorations in this direction are in progress.

Miscellaneous

1. Evidence has been obtained that the mechanism of catecholamine depletion produced in animal tissues by metaraminol is not that of stoichiometric (mole for mole) displacement as some have claimed.
2. SU 5184, an analogue of reserpine which produces selective depletion of catecholamines in the tissues of animals, has been administered to hypertensive patients in oral doses as high as 96 mg/day. No effects were observed and studies have been discontinued.
3. Effective chronic medical therapy for pheochromocytoma using phenoxybenzamine has now been continued for periods of 2-3 years in three patients.
4. A syndrome simulating pheochromocytoma has been recognized in patients with angina pectoris. It is postulated that pressor crises are due to reflexes triggered by reductions in coronary flow.
5. A variety of sympatholytic agents have been found to be useful in treatment of angina pectoris when drug dosages are geared to reduction of cardiovascular responses to exercise.

CARDIOLOGY BRANCH

Introduction

The ultimate goal of the research efforts of the Cardiology Branch, NHI, is to obtain a detailed appreciation of the pathologic physiology which characterizes various forms of clinical heart disease. It is believed that this information will allow the development of more effective approaches to treatment. It is becoming evident that in order to achieve this objective not only are careful clinical and hemodynamic studies of patients with various cardiac abnormalities necessary, but a substantially

greater understanding of the normal myocardial contractile processes is required. Unless the mechanical and energetic processes involved in normal cardiac contraction can be described accurately and in detail, any analysis of the diseased heart muscle will of necessity be quite superficial. In view of this consideration, attention is being directed, in the research program of the Branch, to normal and abnormal hearts; both man and a variety of experimental animals are being studied, and work is carried out on intact hearts as well as on isolated portions of heart muscle, as the experimental situation demands. It is hoped that this non-structured, flexible approach to the study of the circulation will ultimately provide additional information concerning the abnormalities of function which occur in clinical heart disease and will thereby permit a rational approach to treatment.

Determinants of Myocardial Energy Exchange

A study has been initiated in order to define the O₂ requirements of the myocardial membrane activity responsible for de- and repolarization. "Electromechanical dissociation" is produced in isolated canine hearts by perfusing the hearts with blood which has been rendered Ca-free. In these hearts the electrical response is not associated with any contractile activity. Increases in the frequency of depolarization have thus far been accompanied by small but consistent increases of myocardial O₂ uptake, averaging 0.4 μ l O₂ per depolarization per 100 grams of heart. These increases are considered to represent the maximum amount of O₂ that may be required for a depolarization and repolarization and approximate only 1% of the O₂ consumed by the normally contracting heart.

It has been widely held that tension and the time it is maintained, the "tension-time index", is the major, if not the sole determinant of myocardial O₂ consumption. However, in view of studies on skeletal muscle by A. V. Hill in which it has been shown that the rate with which energy is liberated by a muscle is proportional to the maximum velocity of muscle shortening, the possibility was considered that the velocity of myocardial shortening might be an important determinant of myocardial O₂

consumption. In order to explore this possibility, the O₂ consumption of the dog heart was measured under circumstances in which the stroke volume, aortic pressure, and heart rate could be held constant while the velocity of myocardial contraction was augmented by distinctly different inotropic interventions; norepinephrine, calcium, sustained postextrasystolic potentiation, or a combination of these influences. It was found that at a constant level of ventricular work O₂ consumption is correlated with the velocity of ventricular contraction. This was true regardless of the specific inotropic influence by which the velocity was augmented. In addition, this augmentation occurred consistently despite concomitant decrements in the "tension-time" index. It has been concluded that in addition to factors related to force or tension, intrinsic velocity of the myocardium is an important factor in determining myocardial O₂ consumption. Furthermore, it now appears likely that the so-called O₂ wasting effect exerted by norepinephrine on myocardial metabolism may be explained largely by an increased velocity of contraction.

Other evidence has also been obtained that the increase of myocardial O₂ uptake produced by catecholamines is due primarily to the augmentation of hemodynamic activity which the amines induce. Using isolated canine hearts, the response of O₂ uptake to graded doses of isoproterenol, norepinephrine, and epinephrine was determined before and after the induction of arrest with potassium. Increases of O₂ uptake did occur in the arrested state, but were only a small fraction (5 to 20%) of the increases produced by the same doses of amines when the hearts were beating. From these observations it appears that although large doses of catecholamines can stimulate oxidative metabolism directly, the increases of O₂ uptake which catecholamines produce in a beating heart are due primarily to the augmentation of myocardial contractile activity which the amines induce.

Previous observations in experimental heart failure in animals have indicated that there may be a bioenergetic defect in the myocardium involving an impairment of oxidative phosphorylation. This important problem was

studied in myocardial tissue removed from patients with heart failure at the time of cardiac operations. Mitochondria isolated from left ventricular papillary muscles were found to be functionally and anatomically normal. Myocardial creatine phosphate was also found to be within the normal range in these patients. It is concluded that the formation of chemical energy is not impaired in the failing heart, and it is suggested that the biochemical abnormality responsible for defective myocardial function involves utilization of energy in the contractile process.

Mechanics of Myocardial Contraction

EXPERIMENTAL. Continued effort has been directed towards defining the ultrastructural basis of contraction in heart muscle and towards understanding the structural determinants of contraction in the intact heart. Earlier observations on the sarcomere length-tension relation established for the papillary muscle have been extended to the intact heart. Intact left ventricles of both cats and dogs were fixed for electronmicroscopic analysis at known volumes and pressures, and the length of the sarcomeres subsequently determined. These studies have shown that sarcomere length is a function of ventricular pressure, and is independent of absolute ventricular volume. The sarcomere length-tension curve is thus a generalized phenomenon and serves to explain the ubiquitous nature of the Frank-Starling relation in mammals. At intraventricular pressures approximating the upper limits of normal in the functioning heart, sarcomere length corresponds to that seen at the apex of the length-tension curve. Therefore, the limit to ventricular performance seen with increasing initial ventricular volume is determined by the structure of the sarcomere.

A dissociation between thick and thin myofilaments in the sarcomere has also been shown to occur in overdistended ventricles. This observation, along with evidence that slippage of myocardial cells occurs in the overdistended ventricle, helps to explain the mechanical decompensation of ventricular performance which occurs as a consequence of cardiac dilation.

This investigation has recently been extended to the living heart. A technique has been developed which permits nearly instantaneous arrest and fixation of the left ventricle of the dog during any phase of the cardiac cycle. The left coronary artery is cannulated and attached through a sidearm to a powersyringe which suddenly injects glutaraldehyde. Subsequent electronmicroscopic studies have shown that the length of sarcomeres, arrested during diastole are 2.0 to 2.2 microns, while those obtained from hearts arrested in systole measure 1.6 to 1.8 microns. These measurements have allowed direct comparisons with values obtained in the passively distended ventricle, as well as with the sarcomere lengths in isolated papillary muscle. It is anticipated that ultimately the length-tension relationships of the sarcomere of the left ventricle will be known throughout the entire cardiac cycle and under a number of experimental conditions.

The active state of muscle has been defined as a mechanical measure of those processes in the contractile elements which generate force and shortening. In a study of the course of active state in the cat papillary muscle it was found that: 1) In contrast to skeletal muscle, the onset of maximum active state in heart muscle is delayed, developing 100–150 msec. after the first evidence of active state; 2) maximum active state is maintained for about 100 msec. and does not decline until just prior to the development of maximum active tension; and 3) inotropic interventions which alter the contractile state of the muscle such as norepinephrine or changing frequency of contraction, accelerate the onset, increase the intensity, and hasten the decline of the active state. The delayed onset of active state is consistent with the view that following electrical stimulation, an activator is released from the sarcoplasmic reticulum and requires a given time for diffusion to the contractile sites.

The inverse relation between force and velocity of shortening comprises one of the most fundamental properties of the contractile system of muscle. However, it has not been determined whether the force-velocity relation pertains during the course of contraction while changes in muscle length occur. Using the cat papillary muscle, and following the instanta-

neous velocity of muscle shortening during afterloaded isotonic contractions, evidence has been obtained to support the view that the velocity of shortening during contraction depends on instantaneous muscle length during the course of the contraction and is essentially independent of the muscle length at which the contraction began. The extension of these results has permitted the plotting of the force-velocity-length relations which serve to define a given contractile state of the muscle and allow for a more generalized analysis of contraction in heart muscle than is afforded by the relation between initial muscle length and force and velocity.

It is now generally agreed that the myocardium may be considered in terms of an active contractile element arranged in series with a passive elastic component. An investigation was undertaken to define the characteristics of the series elastic element of heart muscle and explore its role in the generation of myocardial force. The stiffness of the series elastic was shown to be a linear function of the developed force of contraction. The load-extension curve of the series elastic was thus exponential in form, the series elastic being stretched an amount equivalent to 8 to 10% of initial muscle length during the development of maximum isometric force. Evidence was also obtained to indicate that the series elastic component is not changed by interventions which greatly alter the activity of the contractile elements such as changing the frequency of contraction or adding strophanthin.

Until recently, the performance of the heart was most commonly described by determining relations between the filling pressure of the ventricle and its stroke volume or stroke work, and by defining alterations in the contractile state in relation to a family of ventricular function curves. However, since it has been shown that the behavior of isolated papillary muscle, like that of skeletal muscle, can be characterized effectively in terms of its force-velocity-length relation, an investigation was undertaken to characterize the performance of the intact canine ventricle in terms of its force-velocity-length relation. Previous difficulties in constructing force-velocity curves were overcome by examining only the first beat after a

sudden alteration in aortic pressure, the change being imposed during diastole. Force-velocity curves were constructed by calculating the velocity of fiber shortening and wall tension at a given systolic volume. It was shown that the force-velocity curves are significantly shifted upwards and to the right by positive inotropic influences and in the opposite direction by acute heart failure. It is suggested that these curves may provide a more unified definition of the contractile state than heretofore possible.

In order to study with greater precision the contribution of atrial contraction to ventricular filling, a highly sensitive, adjustable strain gauge arch was sewn to both atria of dogs, and atrial length-tension curves were determined. It was found that the length-tension characteristics of the atria are similar to those of the ventricle, and that influences which exert a positive inotropic action on the ventricles, i.e., sympathetic stimulation, directly or reflexly induced, and the administration of calcium, isoproterenol, and acetyl strophanthidin, also increase atrial force. Since atrial contraction contributes significantly to ventricular filling, an increased force of atrial contraction may represent another mechanism utilized by the heart to meet increased demands imposed upon it and these studies have clarified some of the determinants of the force of atrial contraction.

It is not known whether an alteration in myocardial distensibility occurs with the development of hypertrophy. It has been the general impression that the hypertrophied ventricle is less distensible than normal, either because of an increase in the total muscle mass or a decreased compliance of individual fibers. This is an important question since hypertrophy is such a fundamental response of the diseased myocardium. Myocardial hypertrophy was produced in rats by subdiaphragmatic suprarenal aortic constriction. It was found that hypertrophied myocardium is similar to normal myocardium in its resting length-tension relationship when suitable corrections are made for differences in muscle mass and length. From these data it would appear that hypertrophy alters myocardial compliance simply by increasing muscle mass, rather than producing a qualitative change in the myocardium.

The effects on the myocardium of paired electrical stimulation were studied in detail. In paired electrical stimulation of the heart, two stimuli are repetitively delivered in such a manner that the second stimulus of each pair is introduced immediately after the termination of the absolute refractory period. If the second stimulus of each pair is sufficiently early, it evokes a propagated ventricular depolarization, but a contraction so weak that it is barely evident. Paired electrical stimuli were applied to the ventricle of dogs with sinus tachycardia. In each experiment the ventricle could be captured by the electrical pacemaker, and the number of effective contractions was reduced from an average of 152 to 109 per minute. When paired electrical stimulation was discontinued, the underlying sinus tachycardia recurred. Similar alterations in rhythm were also induced by paired electrical stimulation in man. Paroxysmal atrial tachycardia and atrial fibrillation were suppressed with paired stimuli delivered through a bipolar catheter in the right ventricle. Paired depolarizations may also be produced by allowing the normal, spontaneous depolarization to act as the first depolarization of the pair. This mode of stimulation has been referred to as "coupled pacing" and reduces the ventricular rate by approximately 50%.

We have termed the augmentation of myocardial performance produced by paired electrical stimulation *electroaugmentation*. The first method we employed for the demonstration of electroaugmentation was measurement of myocardial contractile force. In eight open-chest dogs, in which right ventricular force was recorded with a myocardial strain gauge arch, paired electrical stimuli increased this variable to an average of 275% of the level observed with single impulses delivered at the same rate. In other experiments, carried out on right heart bypass, a change from single to paired stimulation at an identical number of effective contractions per minute markedly improved the contractile state of the left ventricle. The peak rate of rise (dp/dt) of the left ventricular pressure rose, the duration of the systolic ejection period decreased, the mean and peak rates of ventricular ejection and stroke power increased, and the left ventricu-

lar end-diastolic pressure usually fell. Catecholamine depletion with reserpine, or blockade of beta-adrenergic receptors with pronethalol, did not diminish the degree of electroaugmentation produced by paired stimulation, indicating that mobilization of endogenous catecholamines is not responsible for this powerful inotropic response.

Electroaugmentation also occurs in the intact human heart, as shown by investigating the effects of paired electrical stimulation in 17 conscious patients. An increase in the rate of pressure development occurred, associated with a fall in ventricular end-diastolic pressure. Studies on isolated human and cat papillary muscles showed how electroaugmentation shifts the myocardial force-velocity curve upwards and to the right. It was also clear that paired stimulation exerts a positive inotropic effect on human ventricular muscle obtained from patients with congestive heart failure. Analysis of individual contractions indicates that summation of two contractions plays a small, but distinct role in electroaugmentation. No evidence was found to support the contention that the extensibility of the muscle is changed by paired stimulation. Finally, it was shown that merely increasing the frequency of excitation does not account for the electroaugmentation produced by paired electrical stimulation.

The profound metabolic consequences of paired stimulation were observed in experiments in which the myocardial O_2 consumption increased during the augmentation of left ventricular performance associated with paired stimulation, the increases averaging 35%. In all experiments the tension-time indices fell when the stimulation was changed from the single to the paired mode, the decreases averaging 14%.

The effects of paired electrical stimulation on the heart are complex and clinicians will naturally wish to take advantage of the strongly positive inotropic action of this mode of stimulation for the treatment of cardiac failure. It is now clear that paired electrical stimulation is feasible in the human heart and it would seem possible to apply this technique of stimulation on a long-term basis—either by means of an implanted pacemaker delivering suitably

spaced stimuli or by chronic endocardial stimulation. It is also evident that marked increases in the velocity of contraction and in the rate of pressure development may be expected to occur during paired electrical stimulation. However, the clinical benefits that may accrue from this speeding of the contractile process have not yet been defined. It has been disappointing that cardiac output has not risen consistently, even in the conscious patients with myocardial failure. On the other hand, the decline of the elevated ventricular end-diastolic pressure may be of potential clinical benefit, particularly during exertion.

CLINICAL. It is generally agreed that alterations in the rate of contraction constitute one of the most fundamental adaptive mechanisms available to the heart. Almost every major circulatory intervention, be it physiologic or pharmacologic, produces changes in heart rate. A technique was developed for controlling the heart rate in patients with normal atrioventricular conduction by means of an electrical pacemaker which stimulated the right atrium. When the heart rates were increased over a wide range, the cardiac indices remained essentially unchanged. The role of heart rate in the circulatory response to exercise was also examined. When the heart rates were controlled at rates comparable to those achieved spontaneously during exercise, it was observed that cardiac output rose normally with exercise and that this rise was accomplished entirely through an increase in the stroke volume. With isoproterenol infusion, when the heart rate was not permitted to rise, the increases in cardiac output were mediated through increases in the stroke volume. These studies indicate that heart rate plays little role in controlling the cardiac output at rest and that when metabolic demands are increased or the circulation is stimulated by catecholamines, the cardiac output can rise despite a fixed heart rate.

Although it is acknowledged that ventricular end-diastolic size is an important determinant of ventricular performance, until recently it has not been possible to determine the effects of changes in heart rate *per se* on ventricular dimensions in man. At corrective cardiac operation, roentgen-opaque markers were sutured to the surfaces of one or both ventricles in nine

patients. At postoperative cardiac catheterization, cineradiograms were exposed, and the distances between the markers were subsequently measured. As the heart rate was increased by an average of 56 beats per minute, right and left ventricular end-diastolic and end-systolic dimensions decreased significantly. Calculations indicate that the average decreases in external linear dimensions produced by elevation of heart rate in this investigation were equivalent to approximately 35 ml., or about one-half of a normal stroke volume.

The effect of changing heart rate on the force of myocardial contraction has been investigated in several animal species. While some species consistently have shown an increase in contractile force with increasing heart rate (the Bowditch "staircase" effect) a "reverse staircase" or decline in force with increasing rate has been demonstrated in others. Since the effect of changing heart rate on contractile force has not been determined directly in man, a study was undertaken to provide definitive information concerning this relationship. At the time of corrective cardiac surgery a Walton-Brodie strain gauge arch was sutured to the right ventricle of eight patients. While the Bowditch "staircase" effect does not appear to occur consistently in man, a "velocity treppe," i.e., an increase in the velocity of contraction with increasing heart rate, does exist.

The contractile properties of human heart muscle were studied in left ventricular papillary muscles excised from 19 patients at the time of prosthetic replacement of the mitral valve. Analysis of the isometric length-tension relations revealed that the peak of the active tension curve was reached when the muscle was stretched to an average length which exceeded the initial length by approximately 50%. Further increases in muscle length produced a precipitous rise in resting tension, while active tension declined. An inverse relation between afterload and initial velocity of shortening was observed in every muscle, extending to human heart muscle the concept of force-velocity relations. When initial muscle length was increased, isometric tension was augmented but the maximum velocity of shortening remained constant. The addition of strophanthidin or norepinephrine increased the

maximum velocity of shortening as well as the isometric tension, while increasing the frequency of contraction augmented the maximum velocity of shortening only. It is suggested that the contractile state of the human myocardium can be described by the force-velocity relation.

An analysis of the series elastic component of the human myocardium indicated that this component was stretched an average of 8.4% during an isometric contraction, indicating that significant shortening of contractile elements occurs even in the absence of external muscle shortening. When the frequency of contraction was increased, the duration of contraction diminished, but the velocity of shortening increased reciprocally. The resultant force developed, or extent of shortening, remained relatively unchanged. Thus, at any given muscle length, the improved contractile state of the muscle resulting from increasing the frequency of contraction was reflected by the augmented power developed by the muscle, but was not evident in the external work performed. These studies on the contractile properties of papillary muscles provide a framework for an analysis of the performance of the intact human myocardium in terms of fundamental muscle mechanics.

An investigation has also been carried out to determine whether or not the intact ventricle in conscious human subjects adheres to the basic law of muscle contraction which had been elucidated in various experimental preparations, and whether analysis of the force-velocity relation can be useful in assessing the effects of various physiologic and pharmacologic interventions on the human heart. Utilizing a cineradiographic technique, myocardial force-velocity relations were investigated in patients at the time of postoperative cardiac catheterization. The technique consisted of exposing cineradiograms at 30 frames per second and measuring the velocity of movement of roentgen-opaque markers which had been sutured to the external surface of the ventricles, while simultaneously recording intraventricular pressure. A beat-to-beat analysis of the force-velocity relation was then accomplished by measuring the velocity and the pressure at a constant length point in each contraction. When afterload was augmented with methoxa-

mine or was decreased by impeding venous return with a balloon distended in the inferior vena cava, force and velocity varied inversely. In contrast, norepinephrine, isoproterenol, increasing heart rate by electrical stimulation, and paired electrical stimulation, all augmented velocity at any given pressure. Thus, in intact, conscious man, the heart displays the same reciprocal relation between velocity of shortening and generation of force observed in isolated papillary muscle, and a change in the contractile state of the human heart is manifested by a shift in the force-velocity relation.

The response of the heart to exercise has also been analyzed by these methods. Because of the complex nature of the response of the heart to muscular exercise, it has been difficult in intact human subjects to delineate directly the roles played by the Frank-Starling mechanism and by alterations in contractility. When exercise was performed while heart rate was kept constant by electrical stimulation, ventricular end-diastolic dimensions increased significantly. This augmentation in size contrasts with the diminution previously noted during exercise, when heart rate was allowed to rise. Therefore, the importance of the Frank-Starling mechanism in the exercise response was made evident during electrical stimulation. Furthermore, the force-velocity curve shifted so that for any given pressure the velocity of shortening increased, indicating that myocardial contractility had improved. Thus, the normal human heart can utilize several adaptive mechanisms during exercise. These include an elevation of heart rate, the Frank-Starling mechanism and an increased contractile state of the myocardium. It was then shown that following beta-adrenergic blockade with propranolol the augmentation of the contractile state of the heart normally seen during exercise is completely blocked. Nevertheless, an increase in cardiac output is still observed which is mediated through the Frank-Starling mechanism.

There has long been need for a practical method which would allow assessment of myocardial contractility in intact human subjects. Although the peak rate at which intraventricular pressure rises (dp/dt) directly reflects the contractile state of the myocardium, this variable is also altered by changes in both initial

size of the ventricle and by the afterload. Experiments in isolated muscle show that while dp/dt is dependent on both initial fiber length and afterload, the time from the onset of peak dp/dt ($t-dp/dt$) is constant for any one contractile state. However, inotropic agents which increase contractility characteristically shorten $t-dp/dt$ concurrent with increasing the velocity of myocardial contraction. Therefore, an assessment was made of the usefulness of the combination of two measurements— dp/dt and $t-dp/dt$ in the assessment of myocardial contractility in intact unanesthetized man. Ventricular dp/dt was continuously computed with an electronic differentiating circuit and $t-dp/dt$ calculated from high fidelity pressure recordings obtained at a fast paper speed in a large group of patients. With severe myocardial disease, the most prolonged $t-dp/dt$ intervals were observed, with or without depression of dp/dt . In 15 patients, isoproterenol, ouabain or muscular exercise shortened $t-dp/dt$ of both ventricles while dp/dt rose. These observations indicate that $t-dp/dt$ extends the usefulness of dp/dt as an assessment of myocardial contractility and the combination of the two measurements provides a comprehensive analysis of ventricular performance in intact man.

The function of the human left ventricle during exercise has previously been studied only indirectly, and in a relatively limited number of disorders involving the left side of the heart. Accordingly, an investigation was carried out involving the direct measurement of left ventricular pressure, measured directly by the transseptal, retrograde, or anterior percutaneous approaches. Left ventricular and aortic pressures, cardiac output and O_2 consumption were measured at rest and during exercise on a bicycle ergometer. In patients without clinical or hemodynamic evidence of disease involving the left ventricle, minimal changes in the left ventricular end-diastolic pressures were associated with increases in the cardiac index, stroke volume and stroke work, which were commensurate with the increases in the total O_2 consumption. Using the limits established in these subjects, it has become possible to characterize left ventricular function in a large number of patients with a variety of cardiac abnormalities.

The precise mechanisms responsible for closure of the atrioventricular valves under various physiologic and pathologic circumstances have not yet been completely defined. During recent years, there has been particular interest in the role played by atrial contraction, an interest which stems largely from the reappraisals of atrial function in experimental animals and man, and it has been suggested that the decline in atrial pressure during atrial relaxation plays an important role in closure of the atrio-ventricular valves. In the course of diagnostic studies in which selective angiograms with left ventricular injection were performed, we became impressed with the finding that the presence, or the development, of various cardiac arrhythmias was not necessarily associated with perceptible mitral regurgitation. Accordingly, a review of 500 selective angiograms in which the left ventricle was opacified was undertaken. This review yielded 43 studies in which the valve was competent in the absence of an appropriately timed atrial systole. From these observations, it has been concluded that a properly-timed atrial contraction is not *always* essential for mitral valve closure in man.

Action of Digitalis

In spite of the widespread use of digitalis glycosides in clinical practice, neither the mechanism of action of these drugs nor the relationship between their inotropic and toxic properties are fully understood. Radioautography and electronmicroscopy have been used in order to localize radioactive cardiac glycosides in order to determine on which cellular components these drugs act. Previous investigations have indicated that in acutely digitalized hearts radioactive digoxin-H³ may be distributed diffusely throughout the myocardium, associated with the contractile substance itself without specific localization. Tritium labeled digoxin was administered to cats in graded amounts until electrocardiographic signs of glycoside toxicity appeared. Fifteen to twenty hours later, the animals were sacrificed and segments of ventricular myocardium were prepared for electronmicroscopic study. By radioautographic techniques, digoxin-H³ was lo-

calized primarily in the sarcoplasmic reticulum of the myocardium, especially in the area of the Z line of the sarcomere. Since this membrane system, which intimately surrounds the sarcomere, is thought to be involved in excitation-contraction coupling, it has been suggested that cardiac glycosides may exert their salutary action at this locus. These observations are more in keeping with the physiological actions of cardiac glycosides than previous views of glycoside localization.

A considerable amount of confusion remains as to the effects of digitalis on the normal heart. The analysis of force-velocity relations in intact man receiving glycosides acutely has permitted a critical evaluation of this question. Using the methods for determining myocardial force-velocity relations described above, it has been possible to demonstrate that digitalis glycosides augment the contractile state of the myocardium with a shift in the force-velocity relations of the myocardium and a diminution in ventricular size, at a time when these drugs induced little or no changes in cardiac output.

In order to provide more definitive information concerning the value of digitalis in patients with abnormal hemodynamic burdens, but without heart failure, a study was undertaken to determine whether digitoxin administered to rats before and after the production of hypertension by means of constriction of the abdominal aorta modifies the development of ventricular hypertrophy. When suitable corrections for variation in body weight were made, it was clear that aortic constriction resulted in the development of significant ventricular hypertrophy, which was reduced by digitalis treatment. The incidence of fatal congestive heart failure was also significantly lower in the animals which had been treated with glycosides. By reducing the development of hypertrophy, digitoxin appears to permit the ventricles to sustain an excessive burden with less encroachment on this fundamental reserve mechanism. The results of this study would appear to be of considerable significance when the prophylactic administration of digitalis is considered for patients who have not yet developed heart failure, but who have an excessive load on the myocardium.

Several investigators have proposed that the positive inotropic effect of digitalis on the heart is due solely, or in part, to norepinephrine (NE) released from cardiac stores by the glycosides. This proposal is based on findings of a decreased inotropic effect of digitalis after depletion of cardiac stores of NE by reserpine, or after blockade of adrenergic receptors by methalide or dichloroisoproterenol. In these investigations the drug used for sympathetic blockade of NE depletion has always been present during the study of digitalis effects and it is possible that some of the observations have been due to direct action of the drug rather than the interruption of sympathetic pathways to the heart. Accordingly the effects of the cardiac glycoside strophanthidin were studied in isolated papillary muscles obtained from normal and chronically cardiac denervated cats. Papillary muscles from the latter animals were depleted of NE but exhibited a normal inotropic response to strophanthidin. Accordingly, it is concluded that cardiac NE stores are not necessary for the mediation of the inotropic response to strophanthidin.

The problem of digitalis intoxication remains of considerable practical importance. Indeed, the widespread use of potassium-depleting diuretics appears to have increased the frequency of this condition. To determine whether suppression of the toxic manifestations of digitalis by potassium affects the positive inotropic action of the drug, dogs were infused with ouabain while right ventricular contractile force was measured with a strain gauge arch. When ventricular bigeminy or ventricular tachycardia occurred, KCl infusion was begun until sinus rhythm was restored. It was observed that a plateau in the development of contractile force during continuous ouabain infusion did not usually occur prior to the development of toxicity. It was also noted that suppression of the toxic manifestations by KCl permitted the further development of contractile force if the ouabain infusion were continued. The results of this study refute the contention that the maximum effect of digitalis glycosides occurs with doses much smaller than those producing toxicity, and emphasizes the need for the administration of doses of digitalis which are near toxicity, if the maximum positive inotropic

ic effect is to be attained. It also appears that the simultaneous administration of digitalis and KCl may improve the therapeutic/toxic ratio of the glycosides.

As noted in an earlier section of this report, paired electrical stimulation of the heart is capable of suppressing a variety of cardiac arrhythmias. An attempt was made to determine whether this technique would affect digitalis-induced arrhythmias. Two groups of closed-chest dogs received a constant ouabain infusion until death. Paired stimulation produced a regular and slower heart rate than that observed in the controls, but did not permit administration of a larger dose of ouabain before fatal ventricular fibrillation developed. In animals in which ventricular tachycardia was produced with a single dose of ouabain, paired stimulation suppressed the arrhythmia and maintained arterial pressure, and when it was discontinued after two to six hours, the toxic effects of the glycoside had waned. The technique of paired electrical stimulation may prove useful in clinical digitalis intoxication.

Circulatory Control by the Autonomic Nervous System

The activity of the autonomic nervous system is of fundamental importance in modulating the alterations in circulatory function which occur during muscular exercise and a variety of other stressful situations. Accordingly a detailed comprehension of the operation of this system is essential to understanding cardiovascular control mechanisms.

A depletion of sympathetic neurotransmitter had previously been demonstrated in this laboratory to occur in the heart in some patients with heart failure. Since stimulation of the cardiac sympathetic nerves exerts a positive inotropic influence upon the myocardium, it was of interest to determine if the degree of this depletion of norepinephrine (NE) was associated with the alteration of myocardial function characteristic of the failing heart. The relation between cardiac NE concentration and the functional state of the myocardium was studied in left ventricular papillary muscles removed from patients with congestive heart failure at the time of mitral valve re-

placement. Myocardial function was assessed by determining the maximum isometric active tension which the papillary muscle could develop in an *in vitro* system. A significant positive correlation was observed between the NE concentration and the maximal developed force. It was concluded that NE depletion is associated with defective myocardial function, although no causal relationship between these two abnormalities has been established.

In other studies an attempt was made to assess sympathetic nervous activity in cardiac patients by measuring urinary excretion of NE and comparing this with the cardiac store of NE. It was observed that: 1) congestive heart failure is accompanied by an elevated activity of the sympathetic nervous system, as reflected in increased NE excretion; 2) sympathetic activity can be augmented further by the stress of operation; and 3) this sympathetic overactivity is often associated with a deficit of cardiac NE stores. The influence of this increased activity of the sympathetic nervous system with its associated augmented secretion of the neurotransmitter hormone on the turnover rate of NE was evaluated by comparing the disappearance of radioactive NE from the urine in 5 patients with normal activity of the sympathetic nervous system (urinary NE excretion averaging 26.0 $\mu\text{g}/\text{day}$) and in 4 patients with heart failure and augmented sympathetic activity and NE excretion averaging 73.6 $\mu\text{g}/\text{day}$. DL-NE-7-H3 was administered and the decline of specific activity in urine determined for several days. The decay curves of specific activity in the urine were found to be similar in these two groups of patients. It is concluded that increased adrenergic activity does not appear to affect the turnover rate of the neurotransmitter substance and it is suggested that the rate of formation of NE is not controlled by the rate at which it is secreted from the adrenergic neuron. It seems likely that NE synthesis is relatively constant and that during basal rates of secretion it is synthesized in amounts greater than required, the excessive amine being removed by metabolism within the neurons.

Since clinical heart failure is generally associated with cardiac hypertrophy, the possibility could not be excluded that the lowered NE con-

centration in the myocardium might represent simple dispersion of normal sympathetic nerve endings in an increased muscle mass, without a true depletion of total NE stores. Accordingly, this possibility was examined in experimental heart failure in two mammalian species. In the dog heart failure was produced by creating tricuspid insufficiency and pulmonic stenosis. When sacrificed six to eight weeks later, ascites and hepatic congestion were present in all animals. Both concentration and total amounts of NE in both ventricles were strikingly reduced. The effects of tyramine, whose pharmacologic action is dependent on release of intraneuronal NE, were studied in isolated right ventricular papillary muscle preparations. The maximal increase in contractile force above control averaged 138% in muscles from control dogs and less than 20% in muscles from dogs with heart failure. Similar studies using papillary muscles removed during mitral valve replacement from four patients with heart failure have also indicated a correlation between NE depletion and lack of tyramine response. From this study it was concluded that a true depletion of NE stores occurs in heart failure and that this depletion has striking pharmacologic consequences.

In order to determine the nature of the defect in NE storage, NE retaining microsomal particles were isolated from cardiac tissue obtained from both normal and failing hearts of experimental animals. A defective ability to bind NE within this microsomal particle was found in the cardiac tissue from dogs with heart failure, and studies are in progress to characterize this abnormality.

In order to study the physiologic consequences of the NE depletion in experimental heart failure, the response of heart rate and myocardial contractile force to stimulation of the right cardioaccelerator nerve was compared in a group of normal dogs and a group of animals with heart failure. It was observed that the response of animals with congestive heart failure to cardiac sympathetic nerve stimulation was significantly reduced below that of control animals, indicating that the reduced cardiac NE content observed in the failing heart actually interferes with sympathetic nerve function. Congestive heart failure was

also produced in guinea pigs by supravalvular aortic constriction in order to elucidate the mechanism responsible for the changes in NE content observed. Significant reductions in NE stores in the ventricles occurred in the animals with aortic constriction and heart failure. The turnover of NE in the ventricles was not altered in heart failure but a defect of uptake and/or retention of NE was found to be present and may be responsible for the depletion of the cardiac NE stores.

While it is clear that heart failure depresses the response of the myocardium to sympathetic nerve stimulation and to drugs which act by releasing local NE stores, there has been considerable debate concerning the manner in which NE stores affect the basic contractility of the myocardium. Thus, while the sympathetic nervous system provides a mechanism for augmenting basal cardiac contractility, it is not known if the cardiac stores of NE are necessary to establish normal baseline contractility and to maintain the potential for increasing contractility in response to positive inotropic interventions other than sympathetic stimulation. This question is of particular interest in view of the depletion of myocardial NE stores associated with congestive heart failure. Accordingly, the contractility of papillary muscles obtained from cats with myocardial NE depletion produced by cardiac denervation or reserpine treatment was studied. The maximum isotonic velocity of shortening as well as the maximum isometric tension were not depressed in the NE depleted muscles. It was concluded that basal myocardial contractility and the potential for increasing it are not dependent on an intact cardiac store of NE. Preliminary studies indicate that these NE depleted papillary muscles are also supersensitive to exogenous NE.

The role played by the adrenergic innervation of the heart in the mediation of the cardiac response to muscular exercise was studied by determining the effect of beta-adrenergic receptor blockade on the hemodynamic responses to maximal and submaximal exercise. A group of normal subjects were evaluated during high speed treadmill running, while measurements of cardiac output and heart rate were made before and after pharmacologic blockade of the stimulatory sympathetic nerves

to the heart. Significant reductions in heart rate and cardiac output with identical levels of exercise following nerve blockade occurred. Less severe exercise was evaluated in six subjects with mild heart disease utilizing a fiber-optic catheter technique for measuring cardiac output continuously. These studies also showed a fall in heart rate and cardiac output after blockade of the sympathetic nerves to the heart. It has been concluded that the stimulatory sympathetic nerves to the heart play an important role in mediating the heart's response to exertion.

Considerable effort is being directed toward elucidating the precise mechanisms involved in the control of heart rate. In the past it has been believed that alterations in heart rate are brought about by simultaneous reciprocal changes in the sympathetic and parasympathetic influences exerted on the sino-atrial node. An investigation was therefore carried out to test the validity of this classical concept by defining the roles of the two segments of the autonomic nervous system in the regulation of heart rate. Augmenting arterial pressure above control levels with graded doses of phenylephrine always slowed heart rate strikingly, both in the dog and in man. Although complete sympathetic blockade with guanethidine or pronethalol did not significantly alter the degree of slowing, parasympathetic blockade with atropine or vagotomy essentially abolished this response. Conversely, lowering pressure with intravenous nitroglycerin always raised heart rate in the control state, a response abolished by sympathetic blockade with guanethidine or pronethalol, but not by parasympathetic blockade with vagotomy or atropine. Thus, when arterial pressure rises above control, the slowing of the heart rate is mediated by the parasympathetic nervous system, withdrawal of sympathetic activity playing no detectable role; when pressure falls below control levels, the elevation of heart rate is mediated primarily by the sympathetic nervous system. These findings are not consonant with the traditional concept of control of heart rate which predicates simultaneous reciprocal changes in activity occurring in the two components of the autonomic nervous system.

When normal atrioventricular (AV) conduction takes place, the ventricular contraction rate is limited by the AV nodal system, which has been shown in experimental animals to have a longer refractory period than atrial or ventricular tissue. In spite of the fundamental importance of the refractory period of the AV nodal conduction system, this interval has not heretofore been measured in man, and little information is available concerning its duration and the factors which modify it. A practical method for the measurement of the refractory period of the AV nodal system in conscious human subjects was developed, normal basal values for this period were established and the effects of tachycardia, exercise, atropine, and of various sympathomimetic drugs on its duration were determined. A bipolar electrode catheter was positioned against the right atrial wall and stimulated the right atrium at a constant rate just above the sinus rate. Extra impulses were interposed between the regular stimuli; the time intervals between these extra impulses and the preceding stimuli were then shortened progressively. The time interval at which AV conduction just failed was taken as the refractory period of the atrioventricular conduction system (AVRP). In 20 unanesthetized patients, studied in the basal state, the AVRP averaged 350 msec. Atrial tachycardia and the infusion of phenylephrine prolonged the AVRP while atropine, isoproterenol, and exercise shortened it. Thus, in any given patient the AVRP is affected profoundly by sympathetic and parasympathetic stimuli, and measurement of the AVRP permits quantification of these effects.

The reflex response of the arterial and venous beds of the forearm to changes in posture and cold stimulation were determined in a group of patients with familial dysautonomia, and in a group of normal subjects, with a plethysmographic technique. Tilting to a head-up position resulted in no augmentation of arterial or venous tone in patients with dysautonomia, but resulted in striking elevations of both of these variables in normal subjects. Similarly, cold stimulation did not elicit an increase in arterial and venous tone in the dysautonomic patients, a response which was observed consistently in the normal subjects. The

absence of these vasomotor reflexes provides an explanation for the orthostatic syncope which is an important clinical feature of familial dysautonomia.

Regional Circulations

Coronary Circulation

Previous experiments have suggested that an intrinsic adrenergic mechanism for vasoconstriction exists in the coronary vascular bed. Evidence that the coronary vascular bed also contains an intrinsic adrenergic mechanism for vasodilatation (a beta-receptor system) was obtained by studying the response of canine coronary blood vessels to isoproterenol. In intact animals and in isolated potassium-arrested hearts, this drug always decreased coronary vascular resistance. The resistance change could be blocked by pronethalol and did not depend upon increased myocardial metabolism or a decreased level of myocardial oxygenation.

Limb Circulation

The vascular dynamics of the forearm were determined at rest and after several interventions in normal subjects and in patients with heart failure. At rest, forearm blood flow varied directly with cardiac output, forearm resistance varied directly with total resistance, venous tone varied directly with forearm resistance, and indirectly with cardiac output. Patients with heart failure always had lower values for forearm flow and higher values for forearm resistance and venous tone than did normal subjects. The cold pressor test and leg exercise resulted in an excessive elevation of forearm resistance and venous tone, and leg raising resulted in a reduction of forearm blood flow in patients with failure and an elevation in normal subjects. Reactive hyperemia was reduced in the heart failure patients.

The effects of stimulation of the atria, by electrically pacing the right atrium with a catheter electrode and thereby increasing the atrial contraction rate, on the vascular dynamics of the forearm, and on the total peripheral vascular resistance, were studied. Forearm blood flow became elevated, and arterial and venodilatation occurred, while the total systemic vascular resistance generally increased.

From this investigation it is postulated that there are receptors in the walls of the atria, the stimulation of which are capable of producing vasodilatation of the normal human forearm.

It is now well established that the endogenous nonapeptide bradykinin has important effects on the circulatory system, and, in addition, may play a role in many physiologic and pathologic clinical conditions. The effects of the intravenous injection of synthetic bradykinin on the arterial and venous beds of normal human subjects were evaluated by a plethysmographic technique. Bradykinin produced a decrease in systemic arterial pressure, an increase in forearm blood flow, therefore, resulting in arterial dilatation. Following a brief period of venoconstriction, venodilatation occurred. The former was abolished by adrenergic blockade with guanethidine. From this investigation, it was concluded that (a) the venoconstriction is a compensatory reflex elicited during the fall in arterial pressure, and (b) that the prime effect of bradykinin is one of arterial and venodilatation.

In order to determine if the possible peripheral circulatory actions of the nitrite drugs might play a role in their important therapeutic effects, the responses of the arterial and venous beds of the forearm to sublingual nitroglycerin and inhaled amyl nitrite were determined in a group of normal subjects. Nitroglycerin resulted in dilatation of both the arterial and venous beds, while amyl nitrite resulted in dilatation of the arterial bed but constriction of the venous bed. This latter effect was blocked by the antiadrenergic agent guanethidine, and thus was felt to be a reflex elicited by the profound fall of arterial pressure. In order to determine whether the peripheral actions of nitroglycerin reduced heart size, small silver tantalum markers were sutured to one or both ventricles of 11 patients at the time of cardiac operations. Following recovery from operation, the distance between markers on cineradiograms exposed at 30 frames/sec. was measured before and after 0.6 mg. of nitroglycerin. In all patients nitroglycerin decreased end-diastolic and end-systolic dimensions within 2 to 6 minutes, the changes in end-diastolic volume approximating 30% of the stroke volume. Systolic excursions decreased by an aver-

age of 13.3% for the right ventricle and 9.0% for the left ventricle. From these studies, it is clear that nitroglycerin reduces ventricular dimensions in man, and in view of the known relationships between ventricular volume, wall tension, and myocardial oxygen consumption, favorably influences the balance between myocardial O₂ availability and O₂ requirements, thus explaining, at least in part, its effectiveness in angina pectoris.

Pulmonary Circulation

The control of the pulmonary circulation has always been difficult to study, since before an effect on the pulmonary circulation can be ascribed to any specific stimulus acting on the pulmonary vessels, it must be shown to act independently of changes in the systemic circulation, body position, and phase of respiration. A new technique was devised for assessing regional pulmonary blood flow in man by radioisotope scintillation scanning of the distribution of the lungs of intravenously injected radioactive macroaggregated albumin (MAA). Densitometric quantification of the distribution of MAA in the lung field following injection of acetylcholine into a single pulmonary artery revealed that acetylcholine exerted a vasodilatory effect. A systematic analysis of the effects of a variety of drugs on the pulmonary circulation utilizing this technique is planned.

Improvement of Cardiac Diagnostic Methods

During the past two decades there has been steady improvement in the instrumentation for recording information obtained at cardiac catheterization. With the development of effective methods for image intensification, the improved fluoroscopic image greatly facilitated catheter manipulation, and cineradiography and cineangiography became practical clinical techniques. Concurrently, the perfection of catheter-tip manometers, catheter-tip microphones and oximeters has expanded and refined the data that can be recorded oscillographically. However, it has not been convenient to make precise temporal correlations between the radiographic image recorded on the cine film, and data displayed oscillographically, such as the electrocardiogram, and the intra-

cardiac pressure pulses. A device termed the "Cinetrace" was developed in order to superimpose the data displayed on the cathode ray oscilloscope onto one corner of the cine film. Areas of obstruction to blood flow have been localized precisely by moving the catheter across the obstruction and recording the position of the catheter tip at the instant of pressure change. The technique has been used in the diagnosis and localization of valvular, supravalvular and subvalvular obstruction to right and left ventricular outflow.

The clinical applications of an experimental *in vivo* oximeter system employing a fiberoptic catheter have also been investigated. The instrument permits, for the first time, the direct measurement of blood O₂ saturation at the catheter tip, without the withdrawal of blood. By a change of optical filters, the instrument can be made linearly responsive to indocyanine green dye concentration. An electrode at the catheter tip permits the recording of the intracardiac electrocardiogram and facilitates precise localization of the catheter. The instrument has been found to be useful in facilitating rapid and thorough catheterization studies in patients with left-to-right shunts; it has made possible a study of the phasic changes in pulmonary arterial O₂ saturation during respiration and during the Valsalva maneuver; it has permitted continuous measurement of pulmonary arterial O₂ saturation before, during, and after exercise in the supine or upright positions, and it has permitted a study of the effects of pharmacologic interventions upon this variable.

The inability to excrete ingested Na is one of the most fundamental abnormalities which characterizes the congestive heart failure state. Indeed, most of the symptoms and physical signs which occur in heart failure—exertional dyspnea, orthopnea, edema, venous distention, hepatomegaly and ascites—result in large measure from retention of Na and water. A variety of methods is now available for quantifying certain aspects of circulatory function in man. None of these approaches, however, provides information concerning the patient's ability to excrete ingested Na. Accordingly, a simple oral Na tolerance test, designed to detect and quantify abnormalities of sodium excretion was de-

veloped. Following a 4-day period during which daily Na intake was limited to 10 mEq., 80 mEq. Na was administered daily for 4 days and 150 mEq. for another 4 days. The total urinary Na excretion during the 8 day test period was determined. Thirteen normal subjects excreted between 550 and 734 mEq. Na. Of 41 patients with heart disease, 31 excreted subnormal amounts of Na, between 10 and 550 mEq. and only 10 excreted normal quantities. There was no correlation between the impairment of Na excretion, as estimated by the Na tolerance test, and the etiology of the heart disease, the glomerular filtration rate, or any hemodynamic variable. Striking improvement in Na tolerance followed corrective cardiac operations. The Na tolerance test has been found particularly useful in the evaluation of patients in whom clinical examination and hemodynamic studies gave no conclusive evidence as to the presence or absence of congestive heart failure.

Clinical Studies

The clinical findings in 64 patients with idiopathic hypertrophic subaortic stenosis (IHSS) were analyzed and correlated with the physiologic abnormalities in this disease. The hemodynamic response of the left ventricular outflow tract was determined in a group of patients with IHSS during change in body position. It was observed that leg raising with the patient supine, or tilting to a head-down position reduced the outflow gradient and increased the effective outflow orifice area. In contrast, tilting to a head-up position resulted in an increase in the intraventricular pressure gradient and a reduction of the outflow orifice. These changes are probably related to the production of symptoms in these patients and may be responsible for the orthostatic syncope that is often observed.

The infusion of the cardiotonic agent isoproterenol in patients with different forms of obstruction to right ventricular outflow resulted in an increase of the systolic pressure gradient across the pulmonary outflow tract. A diminution of the effective outflow orifice occurred in patients with idiopathic hypertrophic subpulmonic stenosis and in postoperative patients

with secondary subvalvular stenosis who had undergone successful surgical relief of valvular stenosis. In contrast, however, no decrease in the pulmonic outflow area was observed in patients with valvular pulmonic stenosis. These observations indicate that muscular obstruction to right ventricular outflow is dynamic, that it can vary in severity depending on the heart's catecholamine background, and that the response to infusion of isoproterenol can be used to distinguish muscular from fixed obstruction. The response to isoproterenol thus provides a useful test for assessing the nature of obstruction to right ventricular outflow.

The development of an elevation of the pulmonary vascular resistance is a common complication of severe mitral valve disease, and this abnormality has been attributed to a combination of obliterative changes in the pulmonary vascular bed and pulmonary vasoconstriction. In many patients the elevation of pulmonary vascular resistance is extreme and constitutes the major hemodynamic burden faced by the right ventricle. In view of these considerations, determination of the extent to which the abnormalities in the pulmonary circulation can be reversed by surgical correction of the valvular lesion is critically important, particularly when operation is contemplated in a patient with extreme pulmonary hypertension. To investigate this question, pulmonary vascular dynamics were evaluated both pre- and post-operatively in 31 patients with mitral valve disease in whom the valve was replaced with a Starr-Edwards prosthesis, and whose pulmonary arterial systolic pressures were 50 mm. Hg prior to operation. The average pulmonary arterial or right ventricular systolic pressures declined from a preoperative level of 75 mm. Hg to 39 mm. Hg. The gradient across the pulmonary vascular bed decreased while the cardiac output rose and the average calculated pulmonary vascular resistance fell from 679 dynes-sec-cm⁻⁵ preoperatively to 249 dynes-sec-cm⁻⁵ postoperatively. It was concluded that valve replacement generally results in striking reductions in pulmonary vascular pressures and resistances in patients with serious mitral valve disease and pulmonary hypertension.

A rational decision concerning operation in patients with congenital heart disease and pulmonary hypertension is dependent upon an understanding of the natural history of the disease. Accordingly, serial clinical and hemodynamic findings in patients with systemic-pulmonary communications and pulmonary vascular obstruction are being analyzed in an attempt to understand the natural history of the Eisenmenger reaction. Twenty-two patients with isolated ventricular septal defects and patent ductus arteriosus have thus far been studied. Elevated pulmonary vascular resistance may persist from birth in a large number of these patients. Symptoms often begin during the second and third decades of life and survival to the fourth and fifth decades may be anticipated in the absence of surgical therapy.

Section of Clinical Biophysics

It is the purpose of this section to engage in basic experimental studies of the cardiovascular and pulmonary systems with an ultimate objective of establishing realistic and useful mathematical models of these systems. This implies an active laboratory program with strong computer support. The current activities of this section may be summarized arbitrarily under four headings: Pulmonary Mechanics, Pathogenesis of Emphysema, Myocardial Mechanics and Vascular Mechanics.

Pulmonary Mechanics: Studies have continued in an attempt to define further the physical properties of the pulmonary system. These studies have yielded anatomical and rheologic information which has permitted us to develop what appears to be a very promising mathematical model of the lung. In view of the extremely complex nature of the lung our approach to this project necessarily has been modular. That is, having identified and isolated each of the pertinent components of the system, study of the individual parts then can be pursued relatively independently.

In essence this model consists of an ensemble of expansile airspaces that ventilate through an arborized collapsible conduit system, the final confluence of which is the trachea. On the outer surface of this conduit system, there is a distribution of stress which is determined by

the intrathoracic pressure and forces generated within the surrounding medium. On the inner or intraluminal surface of this conduit system there is another stress distribution which is related to the physical properties of the gas, the flow, and the instantaneous dimensions of the conduit system. These dimensions, in turn, are related to the difference between these two stress distributions. With this model in mind the following questions arise. What is the nature of the extramural stress? What is the nature of the intraluminal stress?

The extramural stress on the conduit system is the algebraic sum of the pressure in the contiguous airspaces plus the stresses generated in the surrounding tissues. A number of indirect techniques have been developed for evaluation of the airspace pressures; however, the stress in the surrounding tissues remains to be evaluated. If it can be determined that the visceral pleura plays an essentially negligible role in the visco-elastic behavior of the total lung system, then measurement of the "transpleural pressure" should reflect these tissue stresses. Therefore, a series of studies have been carried out to evaluate the elastic properties of the visceral pleura. A transverse diameter of exposed lung was continuously recorded with a specially designed electrical recording caliper. Tracheal pressure was monitored simultaneously with pressures from the distal points in the lung near the region to which the legs of the recording caliper were attached. Pressure vs. dimensional relations could then be recorded continuously during various imposed respiratory maneuvers. Following measurement of control pressure-diameter relationships, the pleura was slit circumferentially in planes normal to the lung diameter between the legs of the caliper to relieve circumferential tensions in the pleura itself. Pressure-diameter relationships were determined again following this procedure. It was found that the pleura did not affect the pressure-diameter relationship of the lung. Although there are minor rheologic questions which must be pursued in this area, the important point is that the pleura does not play a major role in determining the stress-strain relationships of the lung. Thus, to a first approximation one may consider the trans-

pleural stress to be identical to the extramural stress on a conduit system.

The distribution of stress on the intraluminal surface of the conduit system is considerably more complex. This stress distribution depends on the complicated pressure-flow relationships that occur in nonuniform conduit systems. This problem occupies the frontiers of current day aerodynamics. There are no mathematical models which describe the pressure-flow relationships within nonuniform boundaries such as those of the bronchial tree. Therefore, it becomes important to establish empirical relations describing pressure-flow behavior in physical models of the pulmonary tree. Glass and plastic tubes scaled to various dimensions similar to those in the pulmonary tree were fashioned in various geometric configurations. The pressure distribution was measured along the length of these conduits at different flow rates using gases of known viscosities and densities. Pressures were sampled with various specially designed exploratory probes. The pressure, the flow, and the site of pressure measurement were continuously recorded on electromagnetic tape for A/D conversion and digital computer processing. A family of equations representing approximate solutions to the Navier-Stokes equation was screened with these data. Two of these solutions were found to represent the aerodynamics of these nonuniform conduits satisfactorily. The parameters of these equations may be expressed in terms of the physical properties of the gas as well as certain dimensions of the conduit system.

Armed with the results of the foregoing two series of studies, i.e. the nature of the extramural stress and intramural stress distributions, it was possible to challenge the proposed mathematical model of the lung. This was approached in two ways: study of physical models and study of human data.

Physical models were constructed to represent various segments of the over-all lung model. Various conduits were chosen that had properties very similar to those of bronchial segments. The pressure-area relationships to these conduits and their dimensions were carefully measured. Then the pressure-flow behavior of these systems was studied under carefully controlled conditions of transmural stress using a

variety of special transducers and recording techniques. Both liquid and gas mixtures were studied in these systems so that a wide range of densities and viscosities could be examined. These data were then analyzed in a nondimensional format such that tubes of various sizes as well as subsequent human data could be viewed in a similar coordinate system. The ability of the mathematical model to predict the behavior of the physical models was extremely encouraging. Moreover, when the nondimensionalized data from the human studies were plotted on the family of predicted curves, equally good agreement was found. For example, data taken from maximum expiratory flow-volume curves of human subjects breathing various gas mixtures fell very close to the predicted curve for the model having bronchi of 1.5 mm I.D.

The agreement between the predicted behavior and observation has provided a good level of confidence in the mathematical model. These studies have been extremely informative in shedding light on heretofore unexplained observations. For example, we now have clear insight into the physical events associated with mechanical autoregulation of flow both in the pulmonary bronchial tree and in various other biological flow systems. It is now possible to interpret such terms as "vascular resistance" particularly in the pulmonary circulation more explicitly in terms of precise physical events. Moreover, the mechanisms underlying the various paradoxical observations regarding venous return to the chest, renal and urine flow, and cerebral blood flow can be explained in unequivocal physical terms.

Although the foregoing should be of interest to the general scientific community, the most immediate interest from our point of view is the possibility that we will be able to develop a realistic mathematical model of the total lung system which can be used in the analysis and interpretation of relatively easily obtained clinical measurements of pulmonary function such as the maximum expiratory flow volume curve.

Pulmonary Emphysema

Two of the many obstacles in advancing our understanding of pulmonary emphysema have

been the lack of an experimental animal and our inability to recognize the early pathologic and physiologic lesion of the disease. There is one suggestion in the literature that rabbits develop pulmonary emphysema spontaneously. Studies in collaboration with the Pathology Branch of the National Cancer Institute are under way to attempt to reproduce this observation. In the meantime studies have been done to develop methods of evaluating pulmonary mechanics in the rabbit since standard methods for these measurements in a laboratory animal do not exist.

A small body plethysmograph has been designed and fabricated in which instruments have been developed for the accurate measurement of instantaneous respiratory flow, volume, and pressure. The plethysmograph itself consists of a rigid plastic chamber in which it is possible to ventilate anesthetized animals artificially. A positive displacement electrical recording spirometer continuously records the volume changes of the animal. Instantaneous flows are measured by a specially designed linear flow resistance device. These instruments were found to be linear, free of hysteresis, and to have a uniform dynamic response through 20 cps. Two large groups of rabbits, one group four months old and the other in excess of two years, have been studied with this device. A detailed analysis of the pressure-flow-volume data from these experiments has not been carried out as yet. However, a preliminary crude analysis indicates a significant differences between the young and old rabbits. If the spontaneous development of pulmonary emphysema in these rabbits can be confirmed both physiologically and pathologically, this will represent a major advance in our knowledge. Both longitudinal and transverse studies of normal rabbit populations will be carried out employing these newly developed techniques. Following this, various experimental interventions will be explored. The first of these will be to examine the effect of various noxious gases and the second to examine the effect of papain an agent known to dissolve the cartilaginous support of the bronchial tree. Hopefully, the mathematical model of the lung described earlier will have been validated by this time so that the data from these studies may be processed in accord-

ance with the model so that the observed changes may be interpreted in terms of altered physical properties and dimensions of the systems.

Myocardial Mechanics

The development of a realistic mathematical model of the heart poses a number of formidable questions. In building this model (just as in building the lung model), it is necessary to view the behavior of the individual components and then the behavior of the entire ensemble. Studies that bear on these two objectives have been designed. The behavior of the components of the system has been explored with various *in vitro* papillary muscle preparations. To date, both cat and rabbit muscle preparations have been developed to permit a precise and continuous measurement of instantaneous muscle length, shortening rate and developed tension as a function of time under carefully controlled conditions. The preliminary results of this study indicate that a force-velocity relationship similar to that for skeletal muscle may exist in the initial phases of the contraction; however, during the latter half of contraction, velocity appears to approach some limiting value, becoming relatively independent of either length or load. Various mathematical models are being developed to describe this behavior which are in some respect similar to Hill's "characteristic equation" for skeletal muscle but, in other ways, quite different.

The integrated behavior of an ensemble of these muscle fibers will depend on the pattern of excitation, the over-all geometry of the heart wall, the stresses that exist across the wall, and finally on the distribution and orientation of the component fibers within the wall. Since the duration of contraction is quite long compared to the time for spread of excitation in a normal heart, the pattern of excitation has been assumed to be a "second order" effect, at least for the time being. Therefore, the major unresolved questions relate to the geometry of the heart wall and to the distribution and orientation of fibers within it.

As an initial approach to the measurement of the gross geometry of the heart, a preparation was designed such that the inflow to the

left ventricle could be rapidly switched from blood to a large volume of liquid plastic material. The separation of a number of points on the ventricular surface was recorded continuously using electrical calipers during a control period and then during the sequence of events following the inflow of the liquid plastic. The heart finally comes to rest, filled with this plastic material which rapidly hardens. To the extent that the recordings of surface geometry remain in the same region after this procedure as they did during the control period, it is assumed that the "fixed" heart has essentially the geometry that it has during the normal beating state. Various dimensions are then measured and recorded. The gross shape of the myocardium has been found to vary somewhere between a cylindrical and an ellipsoidal configuration.

Following these gross studies the heart was fixed, sectioned serially, and examined microscopically. The sections were taken in mutually perpendicular planes so that the orientation and number of fibers at each position could be determined. The approximate distribution of fibers in the ventricle is as follows: The inner 40% of the wall has fibers running in the axial direction, the middle 50% has fibers running in a circumferential direction and the outer 10% again has fibers in the axial direction. Although there is considerable variability in some instances, these patterns are generally well oriented and easily recognizable. These findings are consistent with the physiologic studies described in last year's Annual Report in which it was noted that the heart appears to contract with "two degrees of freedom." The implications of these anatomical and physiologic measurements are of obvious significance in the mechanics of contraction.

Vascular Mechanics

Success in providing a model of the vascular bed depends upon determining valid mathematical descriptions of the component vascular segments. Only when this has been accomplished can one turn to description of the integrated behavior of the system. Therefore, the major effort of this section has been in determining the properties of these small "building

blocks" of the system. The mechanical behavior of a small vascular segment depends on the properties of the vessel wall as well as the underlying flow. Under controlled conditions these two quantities may be evaluated independently. Therefore, one set of studies was designed to examine the laws governing blood flow (the laws of fluid motion) and a separate set of studies was designed to explore the laws governing the geometry and physical behavior of the vessel walls (the boundary equations of the system).

Fluid motion is determined by the physical properties of the liquid and the pressure gradient. This relationship is expressed formally by the Navier-Stokes equations which are complicated nonlinear partial differential equations. Simplified solutions to these equations have been derived. These mathematical models were then tested experimentally both in various flow generating devices as well as in vitro dogs True flow in a pulsating vessel was estimated indirectly using two electromagnetic flowmetering systems. A pressure gradient was measured simultaneously and used as the "forcing function" for the mathematical model of flow. The resulting computed flow was then compared with the estimated flow from the electromagnetic flowmeter systems. The standard deviation of the instantaneous coordinate values of the computed flow compared to the "true" flow averaged about ± 7 cc per sec.

Mathematical models of the vascular fluid boundaries have been developed using data acquired from a number of specially designed transducers which record continuously the instantaneous diameter-length relationships and the longitudinal tethering of various arterial segments. Longitudinal and circumferential stresses and strains were studied to evaluate the visco-elastic properties of the aorta in the three principle dimensions of the system, (longitudinal, radial, and circumferential). Longitudinal constraints of the aorta were studied quantitatively by studying the forces associated with both transient and sinusoidal longitudinal displacements of the vessel segments. The major findings of these studies are that 1) the distensibility of the aorta decreases progressively along its length from root to bifurcation. 2) At any point the elastic properties

are different in the three directions, i.e. the vessel wall in anisotropic. 3) The aorta is constrained from longitudinal motion by an "inertio-visco-elastic body." The mathematical model of this vascular tethering phenomenon has been formulated to include the usual behavior of "real" tissues such as stress relaxation, viscosity, hysteresis, etc. This model has shown surprising agreement with observation.

The foregoing studies clarify the relationships between instantaneous flows, pressure, and vessel dimensions in the circulatory systems of animals. This is of considerable theoretical importance in the field of rheology, fluid mechanics, and circulatory dynamics. It is also of practical importance to the clinical physiologist who can apply these principles to the measurement of instantaneous blood flow from more easily obtained pressure information. These studies are being pursued in an effort to establish progressively more comprehensive models of the circulatory system thereby extending our insight into the mechanisms of normal and "abnormal" circulatory dynamics.

SURGERY BRANCH

The research projects of the Surgery Branch have, as in past years, largely centered around studies of normal and abnormal circulatory physiology, particularly in relation to the effects of surgical treatment in patients with congenital or acquired cardiac malformations. Studies have been made in both the laboratory and operating room, but studies in man have been made with increasing frequency utilizing the system for data acquisition provided in the surgical wing.

More than half of the patients referred to the Surgery Branch now require operations involving the insertion of prosthetic cardiac valves, and a significant proportion of the work of the unit has been allied to this general problem. A previous report has described the unique opportunity provided at operation for the measurement of the magnitudes of forward and regurgitant blood flow in patients with aortic regurgitation. These studies have been extended to include the circulatory dynamics in aortic regurgitation not only under basal conditions, but also during various interventions.

While instantaneous aortic flow is measured with a flowmeter on the ascending aorta, the heart rate has been controlled and altered by electrical pacing. Contrary to general belief, it has not been found that a rapid heart rate is beneficial, in terms of net forward blood flow, to patients with aortic regurgitation. In all patients studied, maximum forward flow occurred at a rate between 80 and 100 per minute. Further investigations are being made concerning the effects on regurgitant flow of inotropic agents, alterations in circulating blood volume, and of increased and decreased peripheral arterial resistance.

In most institutions the use of prosthetic cardiac valves has been complicated by the development of bacterial endocarditis in the early postoperative period. This has not occurred in this Clinic, but three patients developed bacterial endocarditis in the late postoperative period, and in each the infection was fatal. This experience has led to detailed re-evaluation of antibiotic prophylaxis in patients with prosthetic valves, particularly when dental or operative interventions are necessary.

Patients with prosthetic valves in the mitral position are now maintained indefinitely on anticoagulation, but, even so, systemic emboli remain a constant threat to them. When the prosthesis is in the tricuspid position, clot formation and embolization are even more frequent. It is becoming evident that the tendency to thrombus formation varies from patient to patient, and may be measured by determinations of platelet adhesiveness. Adhesiveness is measured by determining the fraction of the platelets extracted from blood as it is passed through a column of fine glass beads. Preliminary information indicates that this study is of prognostic significance, and also that adhesiveness can be greatly reduced by the administration of low molecular weight dextran or the maintenance of a low hematocrit. Experimentally, ball valve prostheses have been inserted into the tricuspid position in a large number of calves. Some animals have received no specific treatment postoperatively, others have been given dextran and Coumadin, and in a third group the valves were coated with a solution of graphite-benzalkonium-heparin. Preliminary

observations indicate that anticoagulation is the most effective means for preventing thrombus formation with the valves in this position. In other studies in dogs, however, in which the coagulation mechanisms differ from the calf, the graphite-benzalkonium-heparin coating seems quite effective. This species difference will be resolved by continuing studies to determine the optimal plan of management for clinical application.

The clinical and hemodynamic manifestations of incomplete persistent A-V canal are now generally recognized but, because the malformation is relatively uncommon, there has been little information concerning the effects of operation on the circulatory abnormalities present preoperatively. The hemodynamic effects of operation were studied in 30 patients in whom operative treatment of this malformation had been carried out. In all, the left-to-right shunt was immediately abolished, but recurred in two patients in the late postoperative period. The operation was uniformly effective in reducing elevated pulmonary vascular resistance, and late studies demonstrated normal mitral and tricuspid valve function in all but two patients. In them, mitral incompetence was of principal importance preoperatively, indicating that prosthetic valve replacement may be necessary when the preoperative assessments indicate severe valvular regurgitation.

Serum hepatitis remains an important complication of open heart surgery, and occurs in approximately 12 percent of patients operated upon in this Clinic. It has not been determined whether gamma globulin may prevent serum hepatitis, as it does infectious hepatitis, and the effectiveness of the agent was assessed in a blind study of 200 patients. Half were given gamma globulin before and 30 days after operation, while the others received no specific prophylaxis. The incidence of hepatitis was similar in the two groups.

A previous report described an experimental study concerning the distribution of blood flow to the lungs after a subclavian-pulmonary arterial anastomosis. This indicated that a major proportion of the blood shunted from the subclavian artery passed to the lung *on the side* of the anastomosis, and that the shunt flow diverted a major part of the right ventricular out-

put to the lung on the *side opposite* the anastomosis. Recently, a new method for quantification of regional pulmonary blood flow in man has been developed. Macroaggregated human albumin, tagged with I¹³¹, may be injected either intravenously or into the aortic root, and the distribution of the particles in the lungs determined by external scanning. In cooperation with the Cardiology Branch, the technique is being applied in a systematic study of the patterns of pulmonary blood flow before and after operation in patients with various congenital malformations. Preliminary studies indicate that the observations made in normal dogs, after the Blalock operation, also apply in children, and that gross differences in the distribution of shunted and nonshunted blood also occur with patent ductus arteriosus.

Idiopathic hypertrophic subaortic stenosis has become a well recognized clinical entity. Previous reports have described the studies indicating that in this disease outflow obstruction is intensified when the volume of the left ventricle is reduced, and its contractile force increased. A number of reports from other laboratories have stated that these mechanisms can cause outflow obstruction within the normal left ventricle of the dog under conditions of hemorrhagic shock and/or the administration of inotropic drugs. This hypothesis was re-examined, and particular attention was given to the methods utilized for measuring left ventricular pressure. It was conclusively demonstrated that the pressure gradient between the aorta and the left ventricle, which is often observed in normal dogs subjected to shock, is the result of artifact, and that there is no valid hemodynamic evidence that outflow obstruction can be induced in the normal left ventricle by any known intervention. The question as to whether factitious ventricular pressures can also be recorded in man is presently under investigation.

The normal patterns and volumes of arterial blood flow have been the subject of intensive study in this and other laboratories, but surprisingly little attention has been given to the flow patterns in the venous circulation. With specially designed intravascular flow transducers, instantaneous blood flow was determined in the venae cavae of dogs and related to

the pressure events in the right atrium and right ventricle. In normal animals, the flow pattern was phasic and least flow occurred at the peaks of atrial pressure. Similar relationships were observed in animals in which tricuspid regurgitation, pulmonary stenosis, or various arrhythmias were induced. Retrograde flow of large magnitude was observed with those interventions which resulted in abnormally high peak atrial pressures. An exceptionally interesting abnormality of venous pressure and flow has been observed in both animals and man in the presence of ascites. The presence of severe ascites results in an elevation of the pressure in the inferior vena cava, while the right atrial pressure usually remains normal. In the dog, hemodynamic and angiographic studies indicate that acute ascites results in significant obstruction of inferior vena caval flow, probably caused by angulation of the cava at the diaphragm. In a limited number of patients similar observations have been made, and in them it has also been observed that variations in body position profoundly affect the pressure gradient when it is present. In continuing studies in both man and animals, the possibility that ascites may perpetuate itself by producing hepatic venous pressure elevation is to be investigated.

It is well recognized that morphine is of great therapeutic value in the treatment of patients with acute left ventricular failure and pulmonary edema, but surprisingly little is known of the mechanisms by which this drug alters cardiovascular performance. In normal dogs it was found that morphine resulted in an increase in myocardial contractile force of more than 50 percent, and also that ventricular function, as evaluated by function curves, improved strikingly after morphine injection. The mean rate of left ventricular ejection and left ventricular dp/dt showed similar improvement. In studies in which venous tone was measured, morphine resulted in the pooling of a significant fraction of the circulatory blood volume, indicating that it also has a depressor effect on the capacitance vessels. The effects of the drug will be further studied in animals in which acute left ventricular failure and pulmonary edema have been produced.

General body hypothermia is often used in the treatment of patients with acute infections, neurologic disorders, or during operations in which a thoracotomy is not performed. In such circumstances the threat of ventricular fibrillation is always present, and there has been renewed interest in methods for protecting the hypothermic heart from this arrhythmia. The ventricular fibrillation threshold was studied in dogs rendered hypothermic with an extra-corporeal circuit, and alterations in the threshold were recorded when the arterial blood pH was normal and when it had been increased by the administration of THAM. When the pH was 7.6 or higher the threshold increased by 50 percent or more, indicating that induced metabolic alkalosis may provide significant protection against ventricular fibrillation during hypothermia.

The effects of coupled and paired electrical stimulation on cardiac performance continue to be of interest in both the laboratory and recovery room. Recent studies have compared the effects of single and paired pacing in animals with complete heart block alone, and in animals with heart block complicated by cardiac failure. Paired pacing dramatically improved the performance of the blocked failing heart, but was little better than single pacing in the animals with heart block alone. The possible usefulness of this method in the treatment of post-operative patients with myocardial failure is under continuing study in both the operating room and intensive care unit.

In collaboration with Battelle Memorial Institute a new biologic adhesive was developed, consisting of a gelatin-resorcinol mixture polymerized with formalin. The material was not found to be suitable, as had been hoped, for the sutureless application of intracardiac prostheses. In another experimental study, however, it was found quite effective as a method for controlling bleeding from cut surfaces of liver or kidney. The adhesive system is undergoing modification so that only a single solution need be applied, and that better tissue bonds can be achieved in the presence of moisture or blood.

Several clinico-pathologic investigations have resulted from the establishment of the pathology unit within the Clinic of Surgery.

Ten patients were studied in whom severe mitral regurgitation suddenly occurred as the result of ruptured chordae tendineae. In contrast to patients with the usual form of rheumatic mitral regurgitation, these patients showed little left atrial enlargement, maintained sinus rhythm, and their symptoms, which appeared abruptly, usually led to death or necessitated operative treatment within a short time. In them, the mitral valves were normal except for the ruptured chordae, and in virtually all the etiology of the valvular malformation appeared to be bacterial endocarditis. In a review of more than 200 hearts of patients with valvular abnormalities, four were found to have severe aortic stenosis resulting from a congenitally malformed aortic valve which consisted of only a single cusp and a single commissure. Both operative and pathologic observations indicate that when such a congenitally stenotic valve is encountered, it is not amenable to commissurotomy since gross aortic regurgitation will certainly result. Excision and prosthetic replacement of the unicommisural valve is the only means by which hemodynamic function can be restored. The carcinoid syndrome is generally well characterized, but the cardiac complications continue to be of interest. Most recently it has been demonstrated that the endocardial fibrous lesions characteristic of the disease are often found within the wall of the right atrium as well as on the tricuspid valve, and this atrial fibrosis may prevent the chamber from expanding normally during right ventricular systole. As the consequence of this decreased compliance, right atrial pressures may be excessively elevated and lead to severe symptomatology in patients with carcinoid heart disease and only trivial tricuspid regurgitation.

GERONTOLOGY BRANCH

Final plans have been completed for the design of the Gerontology Research Building to be constructed at the Baltimore City Hospitals, Baltimore, Maryland. Members of the professional staff have contributed considerable time and effort in working out the design of many special features of the building essential to the development of an effective research program on the phenomena of aging.

Aging in the Human

The series of repeated tests on the group of normal volunteers aged 20-96 years (called the Longitudinal Group) has been continued. Since the beginning of the program in 1958, 508 subjects have joined the group and have been tested once. In this group, 347 have been tested two times, 204 three times, 97 four times, and 25 five times at intervals of 18 months. Twenty subjects in the sample have died but only 7 have resigned from the study.

All information obtained from the histories and physical examinations on each subject has been entered into the data retrieval system, and progress is being made on entering the backlog of numerical data. A major effort is being made to enter all data on punched cards and magnetic tape. Significant new additions to the testing program include the measurement of auditory and visual acuity, intra-ocular pressure by tonography, and the measurement of bone cortex thickness as an additional index of bone mass.

Although a systematic analysis of the longitudinal aspects of the data must await the completion of more testing sessions for each individual, certain aspects of the data already collected have been analyzed for age trends by the cross-sectional method. Results of these analyses (pulmonary function, glucose tolerance, EEG, frequency, learning ability) will be described in detail later in this report under the organ system involved.

Physiological Basis of Behavior

Further evidence shows that frequency of the electroencephalogram (EEG) is a critical factor both in understanding the timing of simple behavior and in interpreting age-associated differences in performance. Current findings indicate that involuntary reactions are inversely related to EEG frequency. In the same way, voluntary reactions were shown to be related to EEG frequency by our previous research. We have now found that latency of blocking of the EEG is positively correlated with EEG period (reciprocal of frequency). Blocking latency increases with age but here again it appears, as it did with voluntary response speed, that differences in EEG fre-

quency which are associated with age may account for this relationship. There is now substantial evidence that frequency of the EEG is a critical factor in the timing of nervous system activity, and in the "slowing down" which is associated with behavior of older persons.

Old subjects show a tendency to be less responsive with respect to evocation of EEG blocking responses than young subjects. This result parallels another current finding that frequency of spontaneous galvanic skin reflexes (responses not associated with specific stimuli) is significantly lower in old persons than in young. Further tests of the generality of these specific findings are in progress.

Frequency of spontaneous galvanic skin reflexes may serve as a physiological index of vigilance. Current research shows that subjects who perform a watch-keeping task exhibit fewer such spontaneous responses in an interval of time preceding a signal that is missed than in the period preceding a signal that is detected.

Age Changes in Psychological Performance

It has been hypothesized that interference from the presentation of other items of a learning task is greater for the old person than for the young, and that this age-related susceptibility to interference accounts for the large age differences found at fast learning paces in earlier studies. However, recent experimental evidence does not support interference as a psychological explanation for age deficits in verbal learning. One of the verbal-learning procedures used in the laboratory can be separated into three time intervals for each aspect of learning: time to inspect the material, time to respond, and time between items. These time intervals were independently varied in a series of experiments. Previous research indicated that old subjects benefited more than the young by increasing the time permitted to respond. Current results suggest that old subjects do not benefit by increasing the time between items. These findings are not consistent with the interference hypothesis.

On a logical problem-solving task requiring analysis and synthesis of information, a previous finding from another laboratory indicat-

ed that the performance of subjects above 60 was inferior to the performance of young subjects. Some of the time intervals of the task were not under the subject's control. Similar problems were presented in this laboratory to men aged 28-81 with all time intervals controlled by the subject. The results were compatible with the earlier findings. Until the late 50's no age differences were found, but after the age of 60 performance on a logical problem-solving task declined even when no aspects of the task were paced.

In perceptual studies, interference from judgments of interpolated stimuli has been shown to be greater for old men than for young. The measure of interference in time judgment was based on the increase in the number of errors in judging a set of short intervals before and after an interpolated set of longer intervals. To explore the nature of this type of interference, groups of old and young subjects were given, in addition to the interference task, another time judgment task which provided measures of accuracy, discriminability, and context effects. The new task required the subject to judge whether 220 visual stimuli were longer or shorter than one second. The first 110 stimuli were equally distributed around one second and the next 110 stimuli were predominantly longer than one second. Accuracy was measured for each subject by the difference between his subjective second (based on the first 110 judgments) and the clock second. Discriminability was measured by the variability of the first 110 judgments. A comparison of the subjective second for the symmetrical set and for the set of predominantly longer intervals provided a measure of context effects due to the shift in the distribution of stimuli. Although no age differences were found for the measures of accuracy, discriminability, and context effects, the pattern of correlations with the measure of interference was different for the two age groups. Interference was related to accuracy and discriminability for the old group, but for the young interference was related to context effects.

Pulmonary Physiology

Spirometry was performed in 410 men who comprise the bulk of the longitudinal studies

population. These men were judged to be free of broncho-pulmonary and muscular disease by medical history, physical examination, chest x-ray and electrocardiography. Details of their smoking habits were obtained from a questionnaire which was reviewed with them. For purposes of analysis, the subjects were divided into four groups: (1) 152 non-smokers, (2) 118 current cigarette smokers, (3) 93 former cigarette smokers, and (4) 47 pipe and cigar smokers (past and present). It was found that there were no current cigarette smokers above age 69. For purposes of comparison, therefore, subclassifications of the other groups were made by eliminating subjects above age 69. The resulting subgroups were comparable with respect to age distribution.

The evidence that cigarette smoking impairs spirometric function was twofold: (1) Covariance analysis indicated that the vital capacity (VC), forced expiratory volume at 0.5 seconds (FEV 0.5), forced expiratory volume at 1.0 second (FEV 1.0), and maximum voluntary ventilation (MVV) of the current cigarette smokers ($N=118$) were significantly lower than those of a comparably constituted group of non-smokers ($N=122$). (2) Multiple regression analysis of the data of the current smokers indicated that, when adjustment was made for interrelated effects of age and height, the greater the consumption of cigarettes (measured as packages per day or packages per day times years) the lower were the observed spirometric values. These regressions were significant for the same parameters as in the covariance analysis.

Similar multiple regression analyses of the data of former cigarette smokers indicated that the greater the number of years smoked prior to cessation of smoking, the lower were the observed spirometric values. In this case, the regressions were significant for FEV 0.5, FEV 1.0, maximum mid-expiratory flow (MMF) and maximum expiratory flow rate (MEFR). In addition, when the number of years from cessation of smoking to testing was analyzed and adjustment was made for interrelated effects of age, height and total consumption of cigarettes (as packages per day times years), it was found that the greater the smoking-free interval, the greater were the observed

spirometric measurements. This was significant for VC, FEV 0.5, FEV 1.0, and MEFR. Pipe and cigar smokers performed as well as non-smokers in all tests.

It is concluded that, within the limitations of a retrospective, cross-sectional study, these findings constitute evidence that: (1) cigarette smoking impairs ventilatory function in subjects who appear healthy upon clinical evaluation, (2) this effect is related to the amount of cigarette consumption and (3) this effect is, in part, reversible upon cessation of smoking.

More definitive evidence must await long term observation of subjects before and after cessation of smoking as is contemplated in the longitudinal studies in progress.

Measurements of the uniformity of distribution of air in the lungs have been summarized for 117 participants in the longitudinal studies program. The lung clearance index, a measure of this uniformity, showed that the distribution of air in the lungs was less uniform in the old than in the young. An explanation of the poorer distribution of air found in the old participants was sought in the results of deep breathing tests. Nine young participants did not improve the uniformity of air distribution in their lungs when they breathed at three times their normal resting level while nine older participants improved the uniformity of distribution of air in their lungs significantly (as indicated by a reduced lung clearance index) when they breathed at twice their normal resting levels. The mechanism of this effect is being investigated.

Renal Physiology

The effects of the ingestion of a number of substances on electrolyte excretion in old and young subjects was determined. The ingestion of ethanol, glucose, galactose and casein caused marked increases in the urinary excretion of calcium and magnesium while potassium excretion fell in young subjects. No consistent changes were observed in urinary sodium excretion. Ingestion of xylose and fat failed to produce similar changes. The changes in divalent cation excretion were independent of variations in the glomerular filtration rate, urine pH, urine flow, urine total solute and carbohy-

drate excretion, urine organic (lactate, pyruvate, urate, total organic acid) or inorganic (phosphate) anion excretion, and levels of serum calcium, magnesium and carbohydrate. The increase in urinary divalent cation excretion is a non-specific effect observed following the ingestion of nutrients capable of being readily metabolized in the kidney.

Most of the old subjects failed to show these increases in divalent cation excretion following oral ingestion of 100 grams of glucose. The older subjects usually had sharp falls in urinary sodium excretion which obscured the increases in divalent cation excretion observed in the young. Periods of fasting of 36 hours tended to reproduce the pattern seen in the old subjects in young subjects. The mechanism of this response and the basis for the age differences are being further explored.

A study has been initiated to compare the ability of young and old subjects to clear an acute acid load through the kidneys. Acute oral acid loads, in the form of ammonium chloride gelatin capsules (0.1 gram per Kg. body weight) have been given to adult male volunteers from 2 to 85 years of age. In all age categories, initial serum pH, pCO_2 and total CO_2 were normal. An approximately equal fall in total CO_2 content of blood serum was observed in all age groups following the acid load. By the end of eight hours the younger subjects had restored their serum total CO_2 levels to normal while the older subjects still had markedly reduced levels of serum total CO_2 . All subjects lowered urine pH to similar levels. The rate of ammonia excretion increased markedly in the young subjects but remained low in the old. These preliminary results indicate that aging is accompanied by a reduction in a metabolic function of the kidney.

Experiments were conducted with dogs to study the mechanism of action of ethacrynic acid on the kidney. Stop flow studies showed an increase in distal minimum sodium concentration and examination of medullary tissue showed a decreased concentration of sodium. These changes, along with studies of concentrating and diluting ability, suggest inhibition of sodium transport in the ascending loop of Henle.

Endocrinology

Research on hypertensive disease has long been hampered by lack of chemical methods for the determination of renin and angiotensin. Heretofore all analytical techniques depended on bioassays, most of which are of questionable specificity and precision.

A physico-chemical method for the measurement of renin and angiotensin in the blood is being developed. The method is based on the double isotope derivative assay which has been used for steroid determinations. Dinitrofluorobenzene (DNFB) has been found an effective reagent for reaction with angiotensin. The procedure is to react the unknown amount of angiotensin with DNFB- H^3 following the addition of C^{14} DNP angiotensin and unlabeled DNP angiotensin as indicator and carrier respectively. The DNP angiotensin is precipitated, washed, extracted and chromatographed on a thin layer plate. The yellow DNP angiotensin spot is eluted and counted for tritium (a measure of the original angiotensin content), and C^{14} (a measure of the overall recovery of DNP derivative which permits correction for the tritium losses). As little as 0.1×10^{-6} gram of the peptide has been measured. The sensitivity of the method has been greatly enhanced by the development of DNFB- H^3 reagent with a specific activity that is 22 times greater than the reagent previously available. Work is now progressing on reducing the blank values which will make it possible to determine amounts of the peptide in amounts of 0.1×10^{-9} grams.

The importance of this project is both theoretical and practical. If successful, this method would be the first isotope derivative assay to be developed for a small polypeptide hormone and would lead to the development of other assays of similar important peptides. A practical renin assay, on the other hand, would be a major step in the everyday hospital differential diagnosis of hypertensive disease, since it would almost certainly permit recognition of unilateral (ischemic) reno-vascular hypertensives. The problem has, therefore, a place in the attack on hypertension, a major age-related disease complex. In addition, renin and angiotensin are intimately related to the secretion of aldosterone,

and adequate methodology is badly needed in this area of physiology and patho-physiology. An additional aspect of the program is a study of the interrelations between angiotensin, renin and aldosterone in normal old persons.

Metabolism

Studies of carbohydrate metabolism in the Longitudinal Group are yielding data for a cross-sectional analysis of results. The objectives of the study are: (1) to describe results of the commonly used tests for diabetes mellitus in a carefully studied group of men over the entire adult age span, (2) to investigate the causes of the decline in glucose tolerance with age, and (3) to discover which procedures will predict best the future development of overt clinical diabetes in patients who will be followed for a long time.

Use of the intravenous glucose tolerance test (IVGTT) in clinical medicine has been inhibited by recommendations for increasingly complex methods of analysis of the curve of glucose concentration following rapid glucose administration. By computer techniques we have shown that performance on the test can be judged as well simply by noting the glucose concentration at 60 or 80 minutes following injection as by any of a variety of complicated mathematical techniques as judged by high correlations between results obtained by different methods of summarizing the data. Performance judged by the simple technique correlates as highly with age ($r=0.60$) as do the complex techniques ($r=0.58-0.62$).

Serum insulin levels by radioisotopic double immuno-assay technique in the IVGTT show (1) initial average values of 10 ± 0.7 (S.E.) μU per ml, (2) peak concentration at 10 minutes of 40 ± 3.4 , and (3) slow decline thereafter to 23 ± 1.9 at 80 minutes. There was no significant correlation between age and plasma insulin concentration at any time during the test despite the markedly slower rate of decline of blood glucose concentration with increasing age. These results imply that the age defect is not secondary to inadequate insulin production, but that decreased sensitivity to insulin might play a role.

A series of oral glucose tolerance tests in the Longitudinal Group shows much greater variability in response than with the intravenous test. The necessity of providing normal standards which take into account the variable of subject age is shown by the fact that in the first 24 subjects over age 60 with no personal or family history of diabetes, 13 (58%) had 2 hour glucose concentrations greater than 120 mg per 100 ml, the commonly accepted upper limit of normal. Serum insulin responses in these subjects are also being examined.

The first series of cortisone glucose tolerance tests (CGTT) have been completed. The test was used because of the exaggerated response of diabetics to steroid administration. Thus, if the factors causing deterioration in glucose tolerance that accompanies aging were physiologically distinct from those factors associated with diabetes, a divergent response to cortisone might have occurred. In fact, older subjects showed as accentuated response to cortisone as do diabetics.

Serum insulin responses to the CGTT show that cortisone increases basal insulin concentrations from 10 to $16 \mu\text{U}/\text{ml}$. After oral glucose administration insulin levels increased progressively to a peak mean value at 80 minutes of 126 ± 8.7 (S.E.) in 60 normal subjects. During this rising phase there was no correlation of insulin levels with age. Mean values at 100 and 120 minutes remained 123 and $126 \mu\text{U}/\text{ml}$, but this apparent plateau is caused by the fact that older subjects in the group tend to have a further increase in insulin concentration while younger subjects tended to have a falling concentration. Therefore, at 120 minutes there was a positive correlation with age; insulin levels increase by $11 \mu\text{U}/\text{ml}$ per decade of adult life ($p < .05$). These results confirm those of the IVGTT in that the decline in tolerance cannot be attributed to inadequate insulin response to hyperglycemia.

A series of nomograms to aid in the interpretation of the clinical tests for diabetes have been prepared. These have been constructed for the IVGTT and the CGTT. They permit rapid determination of the rank of an individual in comparison to his age cohorts. The nomograms consist of 3 linear scales: age, glucose concentration (at 80 minutes in the IVGTT, at

120 minutes in the CGTT), and a probit scale. A straight edge connecting appropriate points on the first two scales intersects the third scale at the percentile rank.

Biology of Aging

Behavioral Changes with Age

Recent gerontological theory has attempted to explain age differences in motivation on the basis of physiological factors and in terms of differences in response to various environmental factors. A general factor termed "rigidity" has also been proposed as an explanatory construct. The program of this section is concerned with (a) determining basic behavioral age differences, and (b) finding environmental factors which reduce age differences. The use of lower organisms such as the rat is necessary for such a program.

It was previously reported that naive adolescent rats (2 mo. old) and naive senescent rats (24 mo. old) obtained very low scores of exploration in an open field and remained within a dark hiding area, in contrast to very young (1 mo. old) or young adult (3 mo. old) rats who obtained high scores for exploration. During the past year it was found that fearfulness of adolescent rats could be lowered with an attendant increase in exploration by means of (1) systematic gentling or (2) testing during the dark portion of the dark-light cycle. However, the scores of senescent rats were not changed by these environment variables. These data indicate that the fearfulness of adolescent rats may be decreased by a variety of environmental variables, while the behavior of senescent rats is more rigid, in the sense that variables which reduce fear in young rats do not induce behavioral changes in senescent rats.

Senescent nondeprived rats were found to make more responses to obtain a palatable food reward than did young rats. The senescent rats would be expected to persist in responses which were not "need" reducing if they were more rigid than the young rats. Senescent rats were shown to retain the habit of responding for reward, while younger rats showed less retention.

Differences in performance between deprived young and senescent rats working for a

food reward (one response results in one reward) have not been observed. Also, tests of retention indicate that rates of response are similar for old and young rats over the series of trials given. However, if the senescent rats were more rigid than the young rats, an interposed stimulus (in this case, a stimulus correlated with food reward) should result in a more permanent change in the behavior of the senescent than the young group. Preliminary experiments have shown that the introduction of such a stimulus during retention tests results in a more permanent change in response patterns for the senescent than young rats, while young rats switch back to a formerly rewarded response on later trials.

If physical stamina decreases with increasing age, an increase in the amount of effort to obtain a food reward should result in increasingly large performance differences between young and senescent subjects. An experiment has been completed in which the number of responses required to obtain a reward was progressively increased. Performance differences between food deprived young and senescent rats became increasingly greater as the number of responses required for the reward increased.

Physiological Systems—Heart

Both the isolated heart-lung preparation and the isolated perfused heart from the rat have been used to determine the functional capacity of the myocardium, expressed as an index of left ventricular work (LVWI) in gm M/100 gm dry heart weight. There was a large difference between the work capacity of hearts from young adult (12 mo. old) rats and senescent animals (24 mo. old). The index for heart-lung preparations from 12 month old animals was 163 ± 13 and for 24 month old animals it was 30 ± 4 . The performance scores for the isolated perfused heart were 68 ± 10 and 9 ± 3 for 12 month old and 24 month old animals, respectively. The work output of fatigued hearts could be significantly restored by adding corticosterone at a concentration of $15 \mu\text{g}\%$ to the blood perfusing the heart. Histological examination of the heart at the end of the experiment failed to show morphological changes sufficient to ac-

count for the large differences in performance between the young and old hearts.

Connective Tissue

In spite of many efforts to determine the effect of age on the structure of collagen, much of the evidence on the formation of cross-linkages has been contradictory. Some of these contradictions have been resolved by the following experiments with rat tail tendon collagen from 1, 3, 12 and 24 month old rats. From ultracentrifugation studies the relative quantities of α -collagen (no cross-links) and β -collagen (consisting of units having two α -strands covalently cross-linked) have been determined in the acid soluble portion of the collagen. The fraction of β -collagen increases greatly between 1 and 3 months, but relatively little change occurs thereafter. From solubilization studies it is apparent that both the rate of solubilization and the amount of collagen solubilized decreases greatly with age. Thus cross-linking in soluble collagen levels off after 3 months, but cross-linking beyond the 2-stranded stage, represented by insoluble forms, appears to continue to increase with age.

These results appear to rule out the simple picture that collagen from all age groups consists of varying amounts of the same kind of soluble and the same kind of insoluble collagen, the latter increasing due to aging as new collagen is laid down over it. Instead, the collagen that is soluble becomes increasingly inaccessible to the solvent, and presumably is trapped in a network of massively cross-linking, insoluble material. The levelling after 3 months in the concentration of β -collagen in the soluble component may indicate that β -collagen has a greater tendency in older animals to cross-link further, resulting in the formation of more of the insoluble material.

Nutrition and Aging

Biochemical parameters have frequently been used to formulate hypotheses for the manner in which dietary restriction prolongs life span. Since it is well known that animals offered a restricted amount of food consume the entire ration within a short period of time

whereas food is available to *ad lib* fed controls for 24 hours per day, it seemed necessary to determine whether differences in the interval of time between feeding and sacrifice accounted for the previously reported differences in enzymatic activities between normal and restricted rats. In addition to the previously employed measurements of hepatic succinoxidase and renal alkaline phosphatase activities, protein synthesis was estimated by the incorporation of C^{14} -glycine into proteins of liver slices. Reduced food intake resulted in the expected increments in the two enzymatic activities and also in a decrement in C^{14} -glycine incorporation. Results obtained when the fasting interval was varied indicated that the biochemical alterations associated with dietary restriction were not due to a displacement in the cyclic variation in metabolic activities as a result of the time of feeding.

In studies to determine the mechanisms responsible for the influence of various environmental conditions on the life span of rotifers, animals maintained at different temperatures were subjected to dietary restriction. The mean life span was shortened by increased temperature and was lengthened by dietary restriction at temperatures of 25°C to 35°C. However, the interval of time between the cessation of egg production and death was decreased by increased temperature but was unaffected by nutrition. Thus, these two variables had preferential effects on different physiological stages in the life cycle of these animals. Therefore, it seems that alterations in life span by these environmental factors is not brought about by the same biological mechanism.

Earlier experimental work showed that cultures of a streptomycin-bleached *Euglena gracilis* remained completely viable up to 12–14 days in the absence of an external carbon source. The cells apparently survive through the formation of cytolysomes in their cytoplasm. This process of autodigestion provides a source of carbon to the cells. In the past year, it has been observed that hydrolytic enzyme activity as exemplified by acid phosphatase, cathepsin and esterase, increases during carbon deprivation. Such enzyme activity correlates with the process of autodigestion. Further, there appears to be a time sequence in the hy-

drolytic enzyme activity with esterase, the first to appear. It is not known yet whether the enzymes are actually synthesized during carbon deprivation or whether an inhibitor (repressor) of their activity is removed at this time. Under electron microscopy, carried out in collaboration with Dr. D. Brandes of the Baltimore City Hospitals, at least one of these enzymes (i.e., acid phosphatase) is seen to localize mainly in the Golgi apparatus and in the cytolyosomes.

This observation led to an attempt to isolate the cytolyosomes. By means of a glass bead disruption technique originally developed in this laboratory for the isolation of mitochondria from "normal" non-starved *Euglena*, cytolyosomes have been isolated in an intact state from carbon-starved *Euglena*. This marks the first isolation of such particles from any cell type. Acid phosphatase activity was preserved within the cytolyosomes during fractionation and histochemical manipulations for electron microscopy. Further, the enzyme was distributed identically as was found in intact cells. The isolation of cytolyosomes will allow a study of their biochemical and morphological properties and, hopefully, will provide an insight into the mechanism of autodigestion by the cells.

Earlier studies on the effect of actinomycin D on carbon-starved *Euglena* led to the conclusion that those ribosomes not digested away during starvation were "stabilized", i.e., remained "programmed" for protein synthesis and therefore could synthesize protein if amino acids in proper form were available. Therefore, if carbon-starved cells were fractionated and the appropriate fractions combined and amino acids provided, cell-free protein synthesis should take place in the test tube. In the last year this was accomplished. Fractions from carbon-starved *Euglena* show protein synthesis at rates similar to the control non-starved cells. It will be of interest to fractionate further the protein synthetic system in order to determine the nature of the components which persist over long periods of time. Another problem to be investigated is whether or not the protein synthesis system becomes oriented in the direction of hydrolytic enzyme production during carbon deprivation in *Euglena*.

The formation of chloroplasts in *Euglena* has been shown by other investigators to be ac-

companied by large scale RNA and protein synthesis. It was felt that study of chloroplast formation in *Euglena* would be useful in elucidating cell control mechanisms involving the formation of specific cytoplasmic structures. It is of further interest that the chlorophyll content of *Euglena* chloroplasts reaches a maximum after the cells have entered the post-mitotic or stationary phase of their growth cycle. Chlorophyll synthesis in these cells is sensitive to such inhibitors as streptomycin and fluorouracil as well as high temperature. In the past year it has been found that streptomycin is most inhibitory under conditions that allow cell division. Further, *Euglena* is permanently bleached at high temperature after about 9 cell divisions in a culture. These results suggest the prevention of replication and subsequent "diluting out" with time of some factor essential for the development of a complete chloroplast. Fluorouracil also inhibits "greening" in *Euglena*.

"Error" Theory of Cellular Aging

One of the current theories of aging proposes that with senescence, alterations occur in the structure of the DNA molecule. This error is transmitted to messenger RNA and ultimately to newly synthesized enzymes. These defective enzymes may be inactive and therefore an accumulation of substrates within the cell may take place. This may stimulate an increase in RNA and protein turnover to compensate for the defective enzymes. If the production of inactive enzymes proceeds to the point that increased synthesis cannot compensate for the error, death of the cell and ultimately of the organism occurs. During the past year studies were initiated to test this hypothesis. Two approaches were used. The first was to examine various tissues of rats for age-associated changes which would be predicted to result from the structural errors proposed in this theory. The second method was to determine whether the experimental introduction of errors into protein molecules would also result in the same biochemical alterations. Data which have been obtained on a small number of adult and old rats indicated age-dependent increases in the catabolism of nucleic acid and protein as

estimated by the activities of hepatic ribonuclease and cathepsin respectively. These results are consistent with the error theory. However, in these same animals age-associated decrements rather than the predicted increments were observed in the incorporation of C¹⁴-uridine into liver RNA, in incorporation of C¹⁴-leucine into proteins of liver microsomes and in the C¹⁴-leucine uptake by liver slices in the absence of added essential and non-essential amino acids. Furthermore, no qualitative changes with age were observed in the pattern of lactic dehydrogenase isoenzymes, which represent structurally different forms of the enzyme. Thus, these latter techniques failed to yield data which would completely support the error theory of aging.

The second approach to testing the hypothesis was carried out by feeding animals diets which contained an analogue of an essential amino acid in order to introduce errors into enzymes. Several preliminary studies have been carried out in which female rats were fed diets which contained various amounts of ethionine, an analogue of the essential amino acid, methionine. The results have indicated that the animals failed to survive when fed a diet which contained 1/2% ethionine. Feeding levels of 1/8% or 1/4% of the analogue to adult rats (10-12 mo. old) resulted in initial losses in body weight which subsequently became stable and had no apparent toxic effect on the livers. The result of biochemical analyses of the tissues were consistent with the proposed hypothesis, i.e., increases in the C¹⁴-glycine incorporation and in the activities of cathepsin and ribonuclease in liver tissue were found in the ethionine-fed animals. However, in a subsequent study carried out on younger (3-4 month old) rats fed 1/8% ethionine, not all of the metabolic patterns compatible with the theory were apparent in the experimental group. Development of fatty livers was indicated by the gross appearance of the tissue as well as by an increased liver weight to body weight ratio in the ethionine-fed group. It was concluded that a higher food intake, and hence higher ethionine ingestion, per unit of body weight in young than in adult animals accounted for the discrepancy between the two studies. Therefore, at

present final proof that age-associated changes are due to errors is not available.

Age Pigment

Studies on the properties of lipofuscin age pigments have been continued, and it has been shown that the so-called lysosomal enzymes (esterase, cathepsin) are not associated with the most highly purified age pigment preparations of human liver. It therefore appears either that the age pigments in this tissue are not of lysosomal origin or have lost enzyme activity. On the other hand, isolated lipofuscin from human and bovine heart does show the presence of esterase and catheptic activity, although many individual particles appear to be inactive.

Mechanisms of Cell Death

During the year, a considerable number of changes occurring at the histological level during the process of cell death and aging have been identified. In collaboration with Dr. Wilder's group at Baltimore City Hospitals, experimental infarcts were produced in a group of 50 dogs. These animals were sacrificed at intervals from 1/2 hour to 20 days after tying off a branch of the anterior coronary artery. The infarcted zone, plus control tissue, was removed and a portion fixed and another portion frozen immediately, following which an extensive battery of histochemical and histoenzymological tests were applied. The most striking early changes occurring in cells during the process of cell death included the disappearance of choline-containing lipids, the accumulation of neutral lipids and phospholipids, the depletion of glycogen and an increase in several enzyme activities, notably succinoxidase, esterase, and phosphatase in association with mitochondria. At later stages of infarction, a PAS positive material appeared (not glycogen) and there was a striking increase in leucine aminopeptidase, and the disappearance of phospholipids.

These findings have furnished the basis for a biochemical study of events accompanying cell death. These studies have been undertaken in collaboration with Dr. Prem Batra of the Aging Research Laboratory, Veterans Administration Hospital, Baltimore, and consist of

measurements of adenine nucleotide levels and phosphorylative efficiencies in mitochondrial preparations isolated from damaged or dying tissues. This work has resulted in the development of a new and specific assay procedure for oxidative phosphorylation, which utilizes a modification of the Strehler-Totter firefly luminescence assay. This procedure permits the measurement of phosphorylative rates and P/O ratios within one to two minutes on individual preparations.

Changes during the aging of the firefly lantern (*Photinus pyralis*) were studied by the same battery of the histochemical tests applied to the cell death studies. Age-related decreases were noted in the levels of succinoxidase, ATPase and ADPase. Drastic decreases were observed in stored glycogen in both the reflector layer and the photogenic layer, which were accompanied by marked declines in RNA concentration, considerable decreases in phospholipids by the luxol and Sudan methods, and declines in the concentration of basic proteins, and in tryptophane, sulfahydra, and R groups. These changes probably reflect, in part, intrinsic age changes and, in part, are a result of the limited feeding habits of the adult *Photinus pyralis*.

Hibernation.—In studies of hibernation, it has been shown that there is a considerable change in the content of "apparent glucose" in hamster erythrocytes after the animals have been cold-exposed for several weeks. About 30 to 45 times more glucose oxidase reacting substance is present in the controls and short-term cold exposed animals than in cold-exposed animals just prior to hibernation and in hibernators. The changes in adenine nucleotides and phosphorylated glycolytic intermediates observed were less substantial when hibernators were compared to controls. Both reduced body temperatures during hibernation and active metabolic controls seem responsible for the changes in glycolytic pools.

In attempts to localize the thermal control center of the brains of hamsters by histochemical means paired hypothalami of hibernating and non-hibernating animals were sectioned simultaneously and serially. These sections were then exposed to a variety of histoenzymological tests. No differences were noted in enzyme con-

centrations between the two groups. However, a series of highly specific enzyme localizations was observed in several of the hypothalamic nuclei. These nuclei included the habenular and interpeduncular and associated tracts which stained much more intensely for esterase than any other neural structure, and the suprachiasmatic nucleus which stained intensely and specifically for alkaline phosphatase. Studies of other species indicate that this enzyme distribution pattern is uncommon and not characteristic of all species capable of hibernation, but rather varies widely from species to species. The significance of these striking local concentrations of enzymatic activity cannot at present be evaluated since the function of these structures has not yet been determined.

Basic Biology

In many instances, investigations of the role of cellular processes in longevity and aging are hampered because of inadequate knowledge about the basic mechanisms involved. Hence, part of the research program of the Gerontology Branch is devoted to basic research in cellular biochemistry and molecular biology.

Intermediary Metabolism

Previous work had demonstrated that the oxidation of acetate-2-C¹⁴ to C¹⁴O₂ occurred more rapidly in senescent rats than in adult rats. In order to explain the molecular basis for the above difference, a detailed knowledge of the mitochondrial reactions is required since they are concerned with the terminal oxidation of acetate.

The studies on the mechanism of oxidative phosphorylation in beef heart mitochondria have been continued. The work has centered on the first site of phosphorylation; viz., that occurring in the segment of the respiratory chain between NADH and cytochrome b with some probing of phosphorylating reactions at other sites. The reactions at the first phosphorylation site may be formally illustrated as in Fig. 1. The goal of the research is to isolate each of the unknown components in order to reconstitute the reaction with purified fractions. The purification of coupling enzyme 1 (CE 1) was reported in previous years. An assay for a sec-

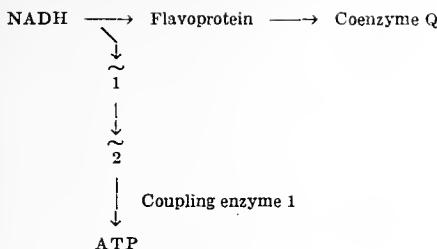


Fig. 1

ond coupling factor has been devised and its purification is in progress. It has been found that submitochondrial particles, depleted of endogenous coupling factors by treatment with urea and supplemented with a saturation level of CE-1, are still deficient in other factors. Under these conditions, additional stimulation of ATP-dependent NAD reduction by succinate (reversed oxidative phosphorylation) was obtained with a crude soluble extract of mitochondria. The new factor is heat-labile, nondialyzable and can be fractionated by precipitation with ammonium sulfate and adsorption on DEAE-cellulose. The preparation of the factor also catalyzes an exchange of ATP with $^{32}\text{P}_i$. However, since the reaction is insensitive to oligomycin, the relationship of this activity to oxidative phosphorylation is not established.

Evidence has been obtained indicating that the flavoprotein shown in Fig. 1 may be identical with the NADH-CoQ reductase whose extraction from mitochondria and partial purification was reported earlier. The CoQ₆ reductase activity and its sensitivity to rotenone appear to be unique to this particular enzyme since preparations of NADH dehydrogenase made by procedures devised in other laboratories gave no activity with CoQ₆. It appears that the activity with CoQ₆ requires preservation of a labile site on the flavoprotein or an additional tightly bound component.

Further evidence that the non-heme iron in the NADH-CoQ reductase is not catalytically active in the oxidation has been obtained using the EPR spectrometer. In collaboration with Dr. H. Beinert, it was found that the enzyme had an EPR signal at $g=4.3$ which disappeared on addition of excess NADH at a very slow rate compared to the turnover rate of the flavin. The high-spin ferric iron responsible for

the signal apparently does not participate in the electron transfer. This confirms previous data obtained by binding the iron with o-phenanthroline.

Recent work has demonstrated that the preparation of NADH-CoQ reductase actually consists of two flavoproteins, both of which have essentially the same activity with menadione but one is twice as active as the other with CoQ₆. Further characterization of the two fractions is in progress.

It has been established in other laboratories that non-phosphorylated high-energy intermediates (analogous to \sim_1 in Fig. 1) generated at the oxidation site between cytochrome c and oxygen (Site 3) may be transferred to Site 1 and utilized to drive the reduction of NAD by succinate. Considerable effort was directed towards separation of soluble factors associated with the reaction since the activity is independent of the terminal CE-1 reaction. It was found that the system, present in submitochondrial particles, was relatively stable and affected but little on exposure to 2 M urea, pH 11, and trypsin. Addition of several types of protein and non-protein extracts and lipids did not stimulate the activity in the treated particles.

An assay specific for the phosphorylation site between cytochrome b and cytochrome c (Site 2) has been devised. It consists of reducing endogenous cytochrome c with ascorbate in the presence of a suitable dye and then driving the electrons to reduce cytochrome b at the expense of energy from added ATP. Specific assays are now available for each site of oxidative phosphorylation.

The energy-dependent cytochrome b reduction had the expected sensitivity to uncoupling agents and electron transfer inhibitors.

Molecular Biology—Properties of DNA and RNA

The ability of copper (II) ions to unwind completely and, under different solution conditions to rewind the two strands of DNA completely, has been proved beyond reasonable doubt, and the mechanism of the denaturation process has been established as consisting of the interpolation of relatively few copper ions between complementary bases and the subse-

quent initiation of a gradual unwinding of the remainder of the double helix. The equilibria involved in these reactions are believed to constitute a model of the unwinding and rewinding of DNA that occurs during its replication in the cell. Copper (II) ions can unwind and rewind the poly (A + U) molecule in the same way as DNA. This reaction is therefore also a model for the attachment and subsequent detachment of messenger RNA from DNA. It should be emphasized that these reactions are models and not biochemical mechanisms since no participation of copper in the biological processes has been demonstrated.

The degradation of RNA by cleavage of the phosphodiester linkages with zinc proved to be non-specific, contrary to the anticipation from the previous finding that the homopolyribonucleotides are degraded at different rates. The reaction is therefore not useful in sequence determinations, although its possible importance

in the biological degradation of RNA should be investigated.

The biological functions of the nucleic acids make it necessary that the following four chemical reactions take place: (1) the formation of phosphodiester linkages, (2) the destruction of these linkages, (3) the ordering of the molecules into helical structures, and (4) the unwinding of these helical structures. It has now been shown that all but the first of these reactions can be brought about by metal ions. Reactions (2) and (3) are mitigated by metal ions binding to the phosphate moieties, whereas reaction (4) is initiated by metals binding to the bases. Conditions have been found under which some metal ions bind to the bases while other metal ions simultaneously bind to phosphate. In view of these findings, it is reasonable to suppose that metal ions can be useful in the regulation of the biological activities of the nucleic acids.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

INTRODUCTION

The past year has seen several changes in the NIAMD, the major one resulting from the untimely death of Dr. Joseph J. Bunim who was both Clinical Director and Chief of the Arthritis and Rheumatism Branch. Dr. Bunim had served as Clinical Director of NIAMD since the first days of Clinical Investigations at NIH. Under his guidance research in arthritis and rheumatic diseases has prospered both at a clinical and basic level. His death removed a backbone of the Institute. We have recently been fortunate in obtaining Dr. Robert S. Gordon, Jr. as Clinical Director. Dr. Gordon has been with the NIH for 14 years and spent three of them as Chief of Clinical Research of the Pakistan-SEATO Cholera Research Laboratory.

Honors have come to many of the staff. The Mead Johnson award to Dr. John Bieri for his work in nutrition, a well deserved honor, may be noted. Dr. Bruce Ames' delineation of the biochemistry and genetics of the pathway for the biosynthesis of histidine has resulted in several honors: The Eli Lilly award of the American Chemical Society and the Biological Sciences Achievement Award of the Washington Academy of Sciences. Dr. Robert Schimke's studies on the control of enzyme levels resulted in the "Outstanding Young Scientist of the Year" award from the Maryland Academy of Sciences. Dr. Leon Heppel's long and distinguished work on nucleic acids resulted in the meritorious Service Medal of the Public Health Service. Superior Service Awards of the Department of Health, Education, and Welfare were received by Drs. N. R. Shulman, C. B. Anfinsen and J. E. Rall.

The staff of an Institute, as has been noted in these reports before, are the very substance of the Institute. It is of interest, therefore, to note a gradual change that has come about at NIAMD as Clinical, Research and Staff Associate positions have become available for physicians, and the Staff Fellowship program has developed for scientists with a Ph.D. degree. Of a doctoral complement of some 234, the senior permanent staff represent 140, or 60%. The Associates number 70 and Visiting Fellows and Scientists, on one or two year appointments, constitute 24. In addition to the above, the scientists in NIAMD have attracted 34 guest workers who came here with support from a wide variety of other organizations. The trend has, therefore, been towards the development of a larger and larger number of scientists here on a temporary basis. As science has become more complex, however, the time for the most profitable post-doctoral training has increased from one to 2 years and increasingly we see 3 years as a better period. The associate positions are generally for 2 years and the staff fellowship for 3 years.

Concomitant with this change towards a larger temporary post-doctoral population has been the development of a more and more comprehensive educational program. The graduate program at NIH lists 99 courses offered, ranging from cultural anthropology to chemical quantum mechanics. There are, in addition, several dozen tutorial seminars for research associates held each semester. Hence, the increased proportion of younger temporary investigators at NIH has been accompanied by an increase in educational opportunities. These trends seem favorable omens for lively research.

OFFICE OF MATHEMATICAL RESEARCH

Work has continued within the areas and along the lines indicated in previous summaries. While new studies, approaches and methods have been initiated, in general the work of the past year can be subsumed under one or both of these categories: (i) generalization and extension in depth of ongoing studies in selected areas of mathematical biology and related mathematics; (ii) application, often concomitant with further development, of methods, models and theories previously developed to particular biological problems.

A new theoretical approach has been developed to define information content of a given set of data relative to a class of models. The method leads to criteria for the design of new experiments to select a given model or to improve estimation for a selected model. A method of inference for assigning an individual to one of two alternative populations has been extended to give an assignment to a hypothetical population "lying between" the two populations thus yielding a quantitative measure which may be viewed as the "degree of transition" of an individual from one population to the other. Revision and up-grading of the computer program (formerly NIH-OMR9B, now denoted SAAM) has continued. A program has been developed for the analysis of logical structure of programs. Collaborative studies have continued in the following areas: Iodine kinetics, magnesium kinetics, and calcium kinetics (all in human subjects) and kinetics of iodination of tyrosine *in vitro*. (Dr. M. Berman and Mrs. M. Weiss.)

The development of a family of mathematical models for the transport, diffusion and consumption of metabolites in the blood-capillary-tissue complex has been undertaken. The formulation is designed to take account of geometry of the arteriole-capillary-venule system, the flow and diffusion aspects, the kinetics of oxygen-hemoglobin association-dissociation and the kinetics of consumption in the tissue. Along with the analytical formulation computational schemes are being developed capable of processing data arising from actual experiments of various types. For the particular geometry deemed appropriate for skeletal

muscle the analytical description yields a system consisting of an elliptic partial differential equation, a non-linear ordinary differential equation, a set of mixed boundary conditions and an interface condition. This system has been solved by constructing an iterative scheme and solving the partial differential equation through explicit formulation of this fundamental solution. The programming of the iterative method for computer application must be preceded by numerical-analytical studies which are under way. (Dr. J. Gonzales-Fernandez.)

If a cell or tissue maintains a stationary internal concentration of a metabolite which is different from that prevailing in the ambient medium and either no flow occurs or flow in fact occurs against an apparent gradient in concentration, then the possibility of active transport is entertained. In assessing such situations, two experimental facts are often, or even always, relevant: (i) In general only the volume *average* of the internal concentration can be determined although the concentration is certainly non-uniform and direction of flow is determined at any point by the local gradient; (ii) Chemical or physical methodology is such that certain linear combinations of concentrations are determined. The role of these two factors has been treated analytically on the basis of a rather general formulation of the appropriate diffusion-reaction problem. It has been shown that for a metabolite, consumed by one set of reactions and produced by another, appropriate separation of the sites of production and consumption can lead to apparent active transport, in that the *average* internal concentration may exceed that in the medium while flow occurs from medium into cell. A linear combination of concentrations can likewise lead to active transport. This conclusion is but one of several corollaries of a series of theorems establishing properties of linear combinations of solutions of the diffusion-reaction equation appropriate to open metabolizing systems and which defy brief narrative summary. (Dr. John Z. Hearon.)

Mathematical and computer studies on visual systems have continued. Computation of the retinal local-averaging function was reported last year. The computed function has been applied to square-wave data to show that the as-

similation effect in the perception of contrast observed by Helson is consistent with a local retinal averaging in conjunction with a "blurring function" which simulates optical imperfections of the eye. Qualitative agreement between predictions from the model and observed data is excellent and quantitative agreement can probably be improved by taking into account the possible adaptive changes in the retinal averaging function. The Fuortes-Hodgkin model for visual response of the *Limulus* eye has been extended and refined. Marked improvement has been obtained by addition of a second feed-back loop and a different assignment of the source of the originally postulated feed-back loop. (Mrs. R. Marimont, Associate Member, OMR, NIMH.)

Mathematical studies of dendritic neurons have been further developed with special reference to a theoretical model for potentials in the olfactory bulb. Considerable effort has gone towards development and testing this model (this work was done in collaboration with Dr. G. Shepherd, at that time Associate Member, OMR, NINDB). The study is designed to take into account a considerable amount of detailed anatomical and physiological information and to provide a theoretical method for interpretation of recorded voltage patterns. Among the parameters or characteristics included are magnitude of geometric obstacle to invasion of soma and dendrites by antidromic impulse, magnitude of dendritic facilitation, kinetics of action potential and "activity" or "passivity" of the dendritic membrane. Computations from the mathematical description show the complicated interplay of these characteristics in determining whether an antidromic impulse upon reaching the soma will be blocked, be delayed or invade rapidly. Reasonably good fits to experimental data have been obtained and a paper is in preparation. (Dr. W. Rall.)

It is now generally accepted that the ability of the mammalian kidney to produce concentrated urine depends upon some type of counter current multiplier. One question has been whether active transport only in the thick portion of the ascending Henle's loop is sufficient for increasing sodium concentration in the inner medulla, if the parallel counter flow of the medullary vessels is taken into account.

For a two-loop system, this question has been answered in the negative: It has been shown that the maximum interstitial sodium concentration in the outer medulla exceeds any concentration in the inner medulla, both interstitial and counterflow loop. This conclusion has been illustrated by numerical solution for typical cases and the work has been submitted for publication. This analysis has been extended to systems of any number of loops. By a rigorous but qualitative argument, it can be shown that a large class of systems cannot concentrate outside the region of active transport. While the mechanism of renal concentration remains unspecified, these results, by ruling out a large class of systems, sharply narrow the range of possibilities. (Dr. J. Stephenson, Associate Member, OMR, NHI.)

LABORATORY OF BIOPHYSICAL CHEMISTRY

The main interest of this laboratory is the study of the relationship of the structure of biologically important macromolecules to function. I would like to illustrate last year's activities of this laboratory by examples.

A special attention was paid to the interaction of the enzyme thrombin with fibrinogen. Thrombin is a highly specific proteolytic enzyme which splits four peptide bonds between arginine and glycine residues in the fibrinogen molecule. In order to come to a better understanding of this reaction, we modified the substrate as well as the enzyme molecules.

Previously we have iodinated tyrosine residues in the fibrinogen molecule and changed lysine residues into homoarginine. This year, fibrinogen was succinylated to change the charge characteristics of the molecule. In this case, it was found that thrombin released only peptide A from the modified fibrinogen, in contrast to the other modifications. (Dr. J. Gladner and Dr. A. Osbahr.)

Chemical modifications usually are very crude. One only occasionally succeeds in modifying selected residues. We, therefore, decided to let nature do the modifications. From kinetic studies, we could convince ourselves that during evolution, both fibrinogen and thrombin underwent modification. In several

cases, the amino acid sequence of the peptides split off by thrombin from fibrinogen are also known. A few years ago, by using synthetic substrates for thrombin, we have found that portions of the substrate far removed from the bond split effected the reaction. From our kinetic studies, coupled with the known alterations nature introduced in the N-terminal portion of the fibrinogen molecule, we were able to deduce that residues removed from the bond split as far away as 18 residues have an effect on the enzyme. This shows that a comparatively large area of the enzyme and the substrate come into contact during the enzymatic reaction. (Drs. K. Laki and N. Chandrasekhar.)

When a protein is synthesized, its amino acid sequence is copied (Translated) from the nucleotide sequence of the messenger RNA. One of the problems is how then the newly synthesized peptide chain takes up its specific three-dimensional structure. One principle seems to be emerging: In watery milieu, hydrophobic residues crowd together and the hydrophylic residues come into the surface. It is quite reasonable to expect that these hydrophylic or polar groups may also form groups and cluster together. (Drs. H. Saroff and W. Carroll.)

Dr. Saroff, in the case of ribonuclease, found evidence for the clustering of polar groups from ion-binding experiments. Because of the clustering, these polar groups collectively, and thus, more strongly, bind ions.

Clustering of the polar groups of course puts great limitation on how the peptide chain can fold. From the consideration of these limitations, it was possible to propose a three-dimensional structure for the ribonuclease molecule. (Dr. H. Saroff.)

The peptides released from fibrinogen have physiological activity. For example, Peptide A released from human fibrinogen acts as vasoconstrictor. It was important to know what portion of the peptide is responsible for this activity. By degradation studies, it was shown that the sequence Asp.-Ser.-Gly. in the peptide is responsible for the activity. (Drs. A. Osbahr, J. Gladner, K. Laki.)

In muscular contraction, the structures we deal with are on an even higher level. Protein molecules in an ordered manner build up the contractile structure which, when the energy is

supplied to it, can shorten and carry out work. In order to come to a better understanding of this process, chemical modifications were carried out on the proteins of the contractile structure. It was found that when the muscle fibers were acetylated or methylated, simple substances such as various salts brought about contraction. In these cases, it is reasonable to conclude that the binding of these ions is responsible for supplying the energy necessary for the work carried out.

There are indications that the binding of these substances leads to changes in the folding of the individual protein molecules that constitute the contractile structures.

These studies are important because biochemistry is still unable to describe in detail, processes where one form of energy is converted to another one. (Drs. W. Bowen and D. Kominz.)

LABORATORY OF EXPERIMENTAL PATHOLOGY

Pathology of Rheumatic Disease

An apparatus (arthrotripsometer) has been developed to determine *in vitro* the frictional characteristics of animal joints whose center of rotation varies throughout an oscillatory cycle. Unlike previously described studies, it provides values at all portions of the cycle of joint motion instead of means. The instrument also senses the deformation of cartilage under different conditions of joint motion and loading. The contours of the friction curves obtained from animal joints differed from those of a metallic (bronze on steel) journal bearing in having more conspicuous variation in the friction coefficients at either end of the cycle of motion than did the latter. The relationship of the friction coefficients of the joints to variations in load and frequency of motion differed from that of metallic bearings and is inconsistent with a simple boundary or hydrodynamic theory of joint lubrication. The data obtained in dog ankles and a metallic bearing indicate that: (1) animal joints had much smaller frictional losses than metallic bearings, even when the latter operated with excellent oil lubricants; (2) despite data presented by others on

artificial bearings, synovial fluid proved to be an effective lubricant in joints. Under conditions of motion restoration of compressed joint cartilage to its normal thickness proceeded much more rapidly than when there was no motion. (Mr. Frank C. Linn.)

Dietary supplements of saturated fats, either in the form of lard or hydrogenated safflower oil, had no demonstrable deleterious effect on the development of degenerative joint disease in male DBA/2JN mice. Although the lard resulted in greater obesity than did the cottonseed oil supplement, the weight gain was more rapid and greater when safflower oil was used rather than 2 hydrogenated products of safflower oil having iodine numbers equal to that of the cottonseed oil and lard, respectively. (Drs. Sokoloff and Mickelsen.)

Scientific Literature Retrieval

The urgent need for development of adequate methods for retrieval of scientific literature as a research tool prompted a literature analysis project to be carried out for the American Rheumatism Association in this laboratory. Three steps were taken to improve the dissemination and retrieval of information on rheumatic diseases: (1) the domain of rheumatology and the limits to which basic research literature should be included in its bibliographic services were delineated by a questionnaire survey; (2) a novel Thesaurus of Rheumatology was prepared to improve the indexing of publications and to codify the vocabulary; (3) a computer based semimonthly publication, the Index of Rheumatology, was developed with the cooperation of the National Library of Medicine. This bibliography is expected to yield approximately 6,000 indexed citations per year. (Miss Ruhl and Dr. Sokoloff.)

It was shown statistically that identification codes for technical (or any other) documents could be assigned at random by the publisher without causing too many cases of confusion. The principal virtue of this method, aside from its simplicity, would be its universality; no convention or centralized authority for assigning code numbers would be required. (Dr. McCutchen.)

Chromosome Studies in Chronic Myelogenous Leukemia

Seventy-three patients referred to or attending the clinics of the Clinical Center of the N.I.H. with the diagnosis of chronic myelogenous leukemia (CML) between May 1961 and October 1963, have been studied as to the significance of the so-called Ph¹ (Philadelphia) chromosome in the marrow cells with respect to the state of the disease. Sixty of the 73 patients were Ph¹ chromosome positive and 13 were negative. The 13 Ph¹ negative cases differed from the Ph¹ positive group by having lower white blood cell and platelet counts and by having a larger proportion of young children under the age of seven. However, the most significant difference was the finding that the Ph¹ negative patients responded poorly to chemotherapy and had a median survival of 18 months as compared to 45 months for the Ph¹ positive population. ($P < 0.1$). The Ph¹ chromosome was observed during all phases of the disease and was unaffected by treatment. In 20% of the Ph¹ positive patients, aneuploidy, mainly hyperdiploid Ph¹ positive cells, was also observed. Two patients had cell lines with 2 Ph¹ chromosomes. In the Ph¹ negative group, there was only one patient with marked aneuploidy. The rest had apparently normal karyotypes in their marrow cells. (Dr. Tjio.)

Characterization and Quantitation of Enzymes in Histochemical Systems

The parameters necessary for quantitation in a histochemical system and for relating the histochemical and biochemical systems have been the subject of continued study. In an effort to determine the relationship of the soluble and tissue-bound (so-called "lyo" and "desmo") components of enzymes in histochemical and biochemical systems, an investigation of the hydrolysis of L-leucyl β -naphthylamide in rat kidney was undertaken. It was found that soluble enzymes were completely extracted from the tissue section during a ten minute incubation. By ultracentrifugation, fractionation procedures and DEAE column chromatography the soluble enzyme was enriched and found to be inhibited by *p*-chloromercuribenzoate and reactivated by cysteine.

The tissue-bound enzyme was solubilized and purified over 1,000 fold by autolysis, fractionation procedures and DEAE column chromatography and its molecular weight and amino-acid composition determined. Electrophoresis of this preparation revealed only two protein bands, both enzymically active. These were separated by elution and found to be isozymes. The tissue-bound enzyme was activated by Co^{++} and inhibited by *o*-phenanthroline, and hydrolyzed peptides, polypeptides and amino acid β -naphthylamides at rates differing markedly from those of the soluble enzyme and the classical leucine aminopeptidase of Smith. A study of these hydrolytic rates revealed it to have the functional capacity of an "aminopoly-peptidase." By the use of a recording micro-densitometer and constant-flow incubating chamber mounted on the microscopic stage, the enzyme in the histochemical section was shown to have the same salient characteristics as those of the purified tissue-bound enzyme. It was concluded that in the system studied the "lyo" and "desmo" components represented in reality distinct enzymes with exclusive characteristics. The fresh frozen section used histochemically appeared to be comparable to the particulate fraction of a $105,000 \times g$ homogenate ultracentrifugate. Zymograms of the whole homogenate demonstrated prominently the soluble enzyme. These studies are preliminary to the comparative evaluation of enzyme reaction characteristics in a histochemical system with enzymes previously characterized by biochemical techniques. (Drs. Felgenhauer and Glenner.)

In addition, by the synthesis and utilization of a wide variety of amino acid and dipeptide β -naphthylamides (Drs. Glenner, Folk, and Cohen), it has been possible to follow by a simple colorimetric assay procedure the purification of a previously undescribed aminopeptidase. This enzyme, termed, aminopeptidase B, has been purified 1,000 fold and has been found to hydrolyze selectively N-terminal L-lysine and arginine residues from peptides and polypeptides. The enzyme is found in the soluble fraction of rat tissue homogenates of duodenum, liver, and pancreas and has been demonstrated to be inhibited by *p*-chloromercuribenzoate and

reactivated by sulphydryl compounds, e.g., cysteine and glutathione.

The biochemical concept of lysosomes (cytoplasmic bodies containing hydrolytic enzymes) has been the subject of extensive electron microscopic-histochemical investigation using as a marker for cytosome (lysosome) demonstration, the sites of hydrolysis of β -glycerophosphate at acid pH. A method has been derived for the successful electron microscopic localization of a further "lysosomal" hydrolytic enzymic activity, that of aryl-sulfatase. Using barium ion as the capture reagent to precipitate sulfate ions released during enzymic hydrolysis of the aryl ester, we have been able to demonstrate the localization of this activity (the type of aryl-sulfatase responsible is as yet unknown) to single membrane bodies corresponding to cytosomes (lysosomes) and cytophagosomes. This represents the first evidence confirming the existence of a "lysosome" enzyme other than acid phosphatase to the cytoplasmic bodies described collectively as lysosomes. Investigations are presently in progress to determine whether the same lysosomes contain both hydrolytic enzymes. (Drs. Hopsu and Glenner.)

Human Lymphocytes in Tissue Culture

MacKinney, Stohlman, and Brecher had shown that when normal human blood is grown in tissue culture with phytohemagglutinin, small lymphocytes can transform into large "blast-like" cells capable of DNA synthesis and division. The present studies were designed to examine the events which precede mitosis of these cells in culture. Combined isotopic and radioautographic techniques revealed that RNA synthesis occurred very soon after the initiation of cultures and preceded DNA synthesis by 24 hours. A marked increase in protein synthesized by the transforming cells was observed in the first 48 hours of culture. The major portion of this newly synthesized protein migrated electrophoretically as alpha globulin; only a minor fraction of it migrated as beta or gamma globulin. Studies are in progress to identify immunoelectrophoretically the specific proteins synthesized and to determine whether their synthesis is a necessary requisite for division. (Dr. Epstein.)

RNA Metabolism in Relation to Steroid Administration

Steroid hormones are known to cause an increase in ribonucleic acid and in protein synthesis. Histochemical investigation of rabbit uterus reveals an increase in the basophilic chromidial substance attributable to an increase in RNA in stromal and smooth muscle cells of the rabbit uterus following treatment with steroids. (Drs. S. N. Cohen [LGAR-NIAID], S. S. Spicer and K. L. Yielding.)

Nucleic Acid and Protein Synthesis

Cytochemical and quantitative autoradiographic studies on the morphology and function of the nucleolus in nucleic acid and protein synthesis have been conducted with HeLa cell cultures. It was found that H^3 -lysine is rapidly incorporated into nucleolar material solubilized either by agents which hydrolyze RNA or by the proteolytic enzyme trypsin. Concentrations of Actinomycin D which inhibit the synthesis of nucleolar RNA also inhibit the incorporation of lysine into nucleolar material but not into extranucleolar chromatin and cytoplasm. When stained for ϵ -amino groups of basic protein with Biebrich scarlet under selective conditions of fixation, nucleoli showing lysine incorporation present a characteristic granular substructure not exhibited or severely altered in cells treated with actinomycin or under conditions of RNA extraction. These findings are interpreted as evidence for the synthesis in the nucleolus of a lysine containing protein which is linked metabolically or chemically to RNA and appears to be independent of chromosomal and cytoplasmic sites of RNA synthesis.

Preliminary results of quantitative autoradiographic experiments with H^3 -lysine, H^3 -arginine, or H^3 -uridine-labeled HeLa cells pretreated with puromycin and Actinomycin indicate that this nucleolar protein is ribosomal in type rather than transfer RNA and that it is demonstrable autoradiographically only under defined conditions of aldehyde fixation. Fixation at suboptimal conditions of temperature, pH and ionic strength or hydrolytic removal of RNA results either in solubilization of some peptide groups in aqueous media or in redistribu-

bution in non-nucleolar areas of the nucleus. It is hoped that this type of investigation may contribute in relating morphologic techniques in pathology and cytology to the problems of molecular biology. (Dr. Suskind.)

An extension of these investigations, an autoradiographic, microspectrophotometric and virologic study, designed to ascertain the sites of viral nucleic acid synthesis and viral protein synthesis of Rous sarcoma virus in transformed (neoplastic) and non-transformed tissue culture cells, is in progress. (Drs. Suskind and Rabotti [LVO-NCI].)

Soluble Ribonucleic Acid (s-RNA)

In collaboration with Dr. Bruce N. Ames (LMB-NIAMD) a technique has been developed for separation of the various amino acid acceptor species of s-RNA by chromatography on DEAE-sephadex. The technique provides as good separation as has been obtained by DEAE chromatography in urea or by gradient partition chromatography, and it is more versatile and convenient than these methods. It is expected that the new chromatographic method will soon be available for the preparation of single s-RNA species and for the analysis of changes occurring in s-RNA which can be related to alterations in the rate and pattern of protein synthesis. (Dr. Smith.)

DNA in Crithidia

In view of the current interest in cytoplasmic organelle replication, a collaborative study was initiated on a hemoflagellate, *Crithidia* sp. *Crithidia* were incubated with H^3 -thymidine for 24 hours and then fixed and prepared for electron microscope autoradiography. A Chalkley grid analysis of the electronmicrographs indicated that the H^3 -thymidine uptake by the kinetoplast, a specialized mitochondrial organelle, was about three times as great per unit random cross-sectional area as that by the nucleus. Practically no background label is found with this technique. These findings add strong evidence that substantial DNA synthesis may occur outside of the nucleus. (Drs. Wetzel, Luke and Greenblatt [LPB-NIAMD].)

Phosphatases on *E. coli*

Cytochemical and morphological studies on *E. coli* have revealed the presence of various phosphatases in different locations within the bacteria. Thus, non-specific alkaline phosphatase has been localized on the surface of the cell in some strains whereas enzymatic hydrolysis of cyclic diester phosphates has been visualized in areas beneath the cell wall. (Drs. B. K. Wetzel, S. S. Spicer and L. A. Heppel [NIAMID-LBM].)

Histidinol Phosphate Phosphatase

The localization of the three phosphate splitting enzymes of the histidine biosynthetic pathway in *Salmonella typhimurium* by electron microscopy could provide information about the compartmentalization of the pathway within the cell. Moreover, the histidine pathway in bacteria appears to be a good system for studying possible improvements in existing phosphatase localization techniques. The activity of one of the enzymes, histidinol phosphate phosphatase, has been studied *in vitro* under conditions required for phosphate trapping on the microscopic level and for fixation of the cells, and an optimal procedure has been established by which about 15% of the phosphatase activity is preserved. Electron microscopic studies are currently in progress. (Drs. Smith, Wetzel and Spicer.)

The interaction of mitochondria and an isolated mitochondrial protein with phenols that uncouple oxidative phosphorylation has been studied. It was concluded that lipophilic reagents such as thyroxine are bound to the protein moiety of mitochondria and are not simply dissolved in the lipid fraction as had been widely assumed. Oxidative phosphorylation can be restored by the addition of serum albumin, a protein which more tightly binds the reagents and thereby removes them from mitochondria. (Drs. Brabus and Weinbach [LPD-NIAID].

Localization of Antigens with Antibody Techniques

The ferritin labeled antibody technique for visualizing specific antigens in cells at the electron microscope level of resolution has been ap-

plied to investigation of the fine structural location of the antigen responsible for the circulating antibody present in patients with myasthenia gravis. This technique has shown localization of ferritin labeled antibody to the H-zone of rat striated muscle. However, the binding of a variety of control conjugates casts doubt on the immunological specificity of this observation and raises question as to the presence in the H-zone of a protein with unique reactivity for ferritin conjugated protein. The relationship of this staining to the immuno-specific staining of striated muscle, observed with fluorescein conjugated antibodies from sera of myasthenia gravis patients remains to be investigated. (Drs. S. D. Douglas, A. J. Gottlieb [LMB-NIAMD], A. J. L. Strauss [DB-NCI], and S. S. Spicer.)

Fluorescein

By means of the Coons' fluorescein labeled antibody technique it has been possible to demonstrate the presence of prolactin in rat pituitary tumors. Fluorescein labeled anti-prolactin also stains prolactin of glands in fish pituitary. The significance of this observation is under investigation. In an extension of the project on visualization of cell antigens by fluorescein labeled antibodies, the site of biosynthesis of growth hormone is being investigated in the pituitary of normal mice and dwarf mice. Fluorescein labeled antibody to glycerophosphate dehydrogenase has also been employed in staining heart muscle and shows the distribution of this enzyme in cardiac muscle fibers. (Dr. E. W. Emmart.)

Fluorescein labeled antibodies to blood group antigens are currently being employed as a means of demonstrating blood group substances in mucus secreting epithelia and connective tissues. Comparison of these results with those of other histochemical staining procedures is being determined to evaluate the histochemical nature of the blood group reactive substances. (Drs. Douglas and Spicer.)

Carbohydrate Histochemistry

A histochemical terminology for mucosubstances has been developed along with a classification of types of mucosaccharides which

can be distinguished histochemically. (Drs. Spicer, Leppi and Stoward.) Current efforts include development of methods for differentiating mucosubstances in tissue sections by light microscopy. A recently developed formazan method reveals differences in mucosubstances not previously observed with conventional PAS and other histochemical procedures. The basis for this differentiation is under investigation. Development of additional methods for selectively localizing carbohydrates and other components in tissue sections are also in progress. (Dr. Stoward.)

Methods have been developed for specifically identifying sulfated mucosubstances histochemically, for differentiating periodate reactive from periodate unreactive mucosubstances, and for demonstrating differences between mucosaccharides on the basis of susceptibility of enzymatic digestion. These methods have been applied to an investigation of the types of mucosubstances present in salivary glands of primates and ungulates. The marked heterogeneity of the various types of secretions in these glands has been recorded and partial characterization of the diverse components has been accomplished including particularly demonstration of periodate reactive sulfomucin in certain epithelia. (Drs. Spicer and Leppi.)

Attempts to specifically visualize acid mucosubstances at the fine structural level have led to the development of a method for staining sialomucins and sulfomucins in mouse epithelia, in addition to the visualization of some connective tissue mucosubstances. This technique involves treatment of sections for electron microscopy with various solutions of ferric chloride at controlled pH levels and provides selective staining of acid mucosubstances in tissue prepared for electron microscopy. (Mrs. Wetzel, Drs. Wetzel and Spicer.)

A battery of histochemical methods has been applied to investigation of the types of secretion in patients with mucoviscidosis (cystic fibrosis) and the presence of sulfomucins as well as sialomucins in the abundant secretions of these patients has been demonstrated. It has also been found that secretions in cystic fibrosis appear to differ only quantitatively and not qualitatively from those in normal controls

resembling closely those in other hypersecretory states. (Drs. Lev and Spicer.)

Application of methods for carbohydrate histochemistry to an investigation of connective tissue mucosubstances has shown a variety of biochemically unrecognized mucosubstances in various connective tissue sites and in particular has differentiated several mucosubstances in the outer, visual layer of the retina. This investigation also has shown precise localization of hyaluronidase resistant mucopolysaccharide (keratan sulfate?) in cartilage, together with an interesting, intimate morphologic relationship of this mucopolysaccharide with sites of calcification. (Drs. Spicer, Horn and Leppi.)

Investigation of the validity of hyaluronidase digestion as a means of localizing mucopolysaccharides histochemically has shown that because of contaminating protease the results are of questionable value when various commercial enzymes are used. (Drs. Leppi and Stoward.)

Histochemistry of Mucopolysaccharides

The epithelium lining the distal bronchial tree and the mesothelium of the pleural surface were studied histochemically for mucopolysaccharides in rabbit, Syrian hamster, mouse, guinea pig and man. In the larger more proximal bronchial radicles, large quantities of sulfated mucopolysaccharides predominated. In contrast, sialomucins in increasing amounts were found more distally along the bronchiolar and alveolar surfaces and the pleural mesothelium. The sialomucin in the distal bronchial tree was increased in the presence of acute inflammation and was absent when chronic inflammation or pulmonary edema was present. It is postulated that the sialomucinous material may have biologic significance because of its bacteriostatic and possible pulmonary surfactant properties. (Drs. Luke and Spicer.)

In an earlier study, evidence was found that a sulfated mucosubstance is a constituent of the fibrinoid thrombi of the generalized Shwartzman reaction, and it was postulated that this mucosubstance contributes to the thrombotic process and may be derived from a sulfated mucopolysaccharide originating in

azurophilic granules of heterophil leukocytes. (Drs. Horn and Spicer.) More recently, a study was initiated on the replication pattern of the several cytoplasmic granules in the granulocyte series. Rabbit bone marrow was incubated *in vitro* with S³⁵-sulfate for varying periods of time and then fixed, sectioned and prepared for electron microscope autoradiography. A preliminary study indicates that the localization of the isotope in heterophil myelocytes occurred initially in the Golgi region and subsequently in azurophilic type granules. (Drs. Horn, Luke, Spicer, and Wetzel.)

Electron Microscopy

THYROID GLAND. A protracted morphological and cytochemical study of rat thyroid glands following administration of thyroid stimulating hormone (TSH) has been concluded. Colloid resorption appears to proceed through the phagocytic engulfment of colloid droplets by apical pseudopods and their subsequent acquisition of acid phosphatase activity. Small, dense, enzyme-rich granules appear to provide the acid phosphatase through fusion with newly formed colloid droplets within 15 to 30 minutes after TSH stimulation. Synthesis of thyroglobulin would appear to proceed via a separate mechanism involving small (0.1μ) vesicles prominent in the Golgi region and beneath the apical plasma membrane of some but not all follicular cells in a given specimen. These studies represent an extension of investigations at the light microscope level which showed engulfment of luminal colloid into thyroid epithelial cell droplets and admixture of both acid phosphatase and esterase with these droplets shortly after stimulation. (Drs. Wetzel, Spicer and S. H. Wollman [LPHY-NCI].)

MYELOID ELEMENTS. Studies on the fine structure and cytochemistry of rabbit myeloid elements are near completion with the goals of more thoroughly characterizing granulocyte development and contributing to an understanding of the nature of their cytoplasmic granules. Rabbit heterophils appear to contain at least three distinct types of cytoplasmic granules. The primary granule, rich in acid phosphatase, and (by correlative histochemical studies) sulfated mucosubstances and basic

proteins, is produced by the earliest identifiable heterophils. Application of electron microscope autoradiography also has afforded a means of confirming the localization of the sulfated mucopolysaccharide in the primary type of granule in rabbit heterophils. Numerous primary granules accumulate as the cells enlarge, but an abrupt shift seems to occur to *de novo* synthesis of alkaline phosphatase-rich secondary granules. Continued cell division probably accounts for the diminished numbers of primary granules present in more mature cells, which contain numerous secondary granules and an additional population of smaller, acid phosphatase-rich tertiary granules. A complimentary combination of morphological and cytochemical characteristics permits this type of analysis. Differing roles of acid phosphatase in granulogenesis are evident in eosinophiles, basophiles, mononuclear cells and platelets. Acid phosphatase-rich organelles, presumably functioning as cytolyticosomes, have been encountered in erythroblasts and reticulocytes at the time of resorption of the chromidial substance and the mitochondria of these cells. Cytochemical studies of heterophils phagocytosing bacteria have demonstrated the contribution of all three types of granules and their phosphatases to the bacteria-laden phagocytic vacuoles. (Drs. Wetzel, Horn, Luke, and Spicer.)

Epinephrine

It was shown previously that exposure of animals to various stresses such as high altitude hypoxia, cold or arduous exercise produces liver glycogen depletion, fatty changes in the viscera and elevations in certain serum enzymes. Since release of epinephrine and norepinephrine from the adrenal medulla is believed to play a role in the response to such stresses, the effects of epinephrine in oil, which is slowly absorbed and has a prolonged action, were studied.

Rats given a large dose of epinephrine in oil subcutaneously developed marked transient fatty changes in heart, liver, kidney and muscle, severe myocarditis, pulmonary hemorrhages with hemoglobin-like crystalline deposits, and renal hemoglobin casts. Liver glycogen was depleted. Serum values of glutamic

oxalacetic and pyruvic transaminases, aldolase and urea nitrogen rose sharply and alkaline phosphatase fell 24 hours after a dose and returned to normal within 72 hours. Pretreatment with Dibenamine hydrochloride, an adrenergic blocking agent, prevented or lessened most pathologic and serum enzyme changes, but not depletion of liver glycogen.

It was concluded that the serum enzyme changes are due to alterations in cellular permeability. This altered permeability is attributed to a relative hypoxia, due to changes in oxidative metabolism with increased oxygen consumption by tissues induced by epinephrine, and to severe local hypoxia, caused by prolonged marked vasoconstriction. The fatty changes, particularly in the heart, are attributed to the effects of epinephrine on fat mobilization and metabolism and the specific avidity of the myocardium for storing catechol amines. (Drs. Highman, Altland [LPB-NIAMD], and Garbus.)

Erythropoietin Production by Juxtaglomerular (JG) Cells

A study of rats exposed to high altitude has revealed additional evidence that the renal JG cells produce erythropoietin. Various investigators have sought such a relationship, but their methods of inducing oxygen deficiency also decreased renal blood volume so that the observed results could have stemmed from either factor. In the present study, rats were exposed to a simulated altitude of 25,000 feet 6 hours daily and 5 days a week for 6 weeks. This resulted in polycythemia without a decrease in renal blood volume. Rats were killed either before or immediately after altitude exposure and the degree of granularity of the JG cells or juxtaglomerular cell index (JGI) was determined by the method of Hartroft and Hartroft. Plasma erythropoietin levels were determined by the bioassay method of Stohlman, and an improved method of extracting erythropoietin from the kidney was devised.

A close correlation was found between the granularity of the JG cells and circulating erythropoietin levels. Thus, after a weekend of rest and before exposure, the JGI was high and the circulating erythropoietin levels were low.

Following exposure, however, large decreases in the JGI were accompanied by high circulating erythropoietin levels. Similar correlations between extractable renal erythropoietin and the JGI were observed.

These findings support the view that JG cells are concerned with production of erythropoietin and that their increased secretory activities play a role in acclimatization to high altitude by increasing the circulating red cell mass through liberation of increased amounts of erythropoietin. (Drs. Demopoulos, Highman, Altland [LPB-NIAMD], Gerving [Univ. of Southern Calif.], and Kaley [N.Y. Univ.].)

Effect of Adrenalectomy on Work Performance

In collaboration with Drs. Maling (LCP-NHI), Altland and associates, studies are nearing completion comparing biochemical and histopathologic changes during exercise between normal intact rats and adrenalectomized, adrenal demedullated and chemically sympathectomized rats. Sympathectomy was produced in adrenal demedullated rats by depleting the stores of catechol amines with syrosingopine or by blocking release from nerve endings with a bretylium-like drug, BW 392C60. Running in a rotating cage, intact rats did not become fatigued in 4 hours, whereas the other groups became fatigued within 90 to 200 minutes. Differences between groups were noted in changes during exercise in levels of plasma glucose, free fatty acid and lactic acid as well as in the triglyceride and glycogen content of various tissues, using both chemical and histologic methods. The differences suggest that the increased susceptibility to fatigue of adrenalectomized and sympathectomized animals is due to impairment in their ability to mobilize adequate energy fuel. (Dr. Highman.)

Dipetalonema witei

In a collaborative study with Dr. Weinstein (LPD-NIAID), it was found that following subcutaneous infection of the jird, a rodent found in North Africa, with *Dipetalonema witei* (a filaria) larvae dissected from a tick, *Ornithodoros tartakovskii*, extensive nervous system symptoms developed in some of the animals. The symptoms ranged from severe limb

incoordination, spastic movements and partial paralysis to complete paraplegia, between 31 to 93 days following infection. Worms (filaria) were found by gross dissection and histopathologic examination in the brain and spinal cord of some of these animals as well as in various other tissues. A histopathologic study is nearing completion to determine how the filaria reach the central nervous system from the site of inoculation in the subcutaneous tissue of the abdominal wall. Although it is not known whether this parasite is pathogenic for man, this study may be important in elucidating the mode of entry of other parasites that attack the central nervous system in man. (Dr. Highman.)

Toxic Compounds

It has been proposed that the more stable iodate salts be substituted for the iodide salt for use in the prevention of goiter in tropical countries. A study was made with Dr. Webster (LBP-NIAMD) on the acute and subacute toxicity of potassium iodate in dogs. In dogs that died or were killed within 24 hours after a large oral dose of potassium iodate, 200-250 mg/kg, there were noted areas of hemorrhagic necrosis in and near the antrum of the stomach, fatty changes in the liver and kidney, depletion of lipid in the adrenal cortex, and less constantly, centrilobular necrosis in the liver and foci of necrosis in the renal cortex and bladder mucosa. One dog, killed about 10 weeks after a dose of 200 mg/kg, showed degenerative changes in the retina. Subacute toxicity was studied in 4 dogs given repeated doses of 6-100 mg/kg KIO₃ for 68-192 days. There were large deposits of hemosiderin, particularly in the spleen, suggesting hemolytic activity by iodate, and a slight to moderate decrease in red and white blood cell counts. Two dogs that received the last dose of iodate on the day of sacrifice showed foci of hemorrhagic necrosis in the gastric mucosa as described above. No retinal degeneration or other significant changes were noted. A report on the chronic oral toxicity of potassium iodate in dogs will follow. A report on these studies was submitted earlier to the Food and Drug Administration. (Dr. B. Highman.)

Cycasin Research

Experimental studies with the glycoside, cycasin, a β -D-glucosyl-oxyazoxo-methane, isolated from the seeds of *C. circinalis* has continued during the past year. The glycoside is highly toxic in rats when taken orally in large amounts producing massive liver necrosis. It is carcinogenic when consumed in small amounts by conventional laboratory animals. Studies in rats indicate that the carcinogenic effect is not limited to one specific site, but that one is dealing with a general carcinogen producing neoplastic disease at various sites simultaneously in a respectable percentage of the animals. Although the development of the acute toxic effects following ingestion is well established for man, it is not known at present whether it is carcinogenic for man.

Recent studies by Dr. Matsumoto indicate that cycasin methylates nucleic acids, and methylation of the guanine in the 7 position has been indicated. Its mutagenic effect is briefly described by Dr. Smith below. Thus far no evidence has been obtained that it is teratogenic. Its radiomimetic effect has recently been noted by Dr. Teas.

During the past year, data have been collected along two main lines of investigation. One dealt with the carcinogenic effect of cycasin after short- and long-term exposures of rats to the glycoside; the other with the excretion pattern of cycasin in germ free and conventional rats, a project summarized below by Dr. Spatz.

A comparative study between short- and long-term exposures of rats to cycasin has yielded the information that long-term exposures (9 months) produced a high incidence of hepatomas followed by renal and intestinal neoplasm in frequency. On the other hand, short exposures (13-21 days) to cycasin predominantly induced kidney tumors followed by hepatic and intestinal neoplasms in frequency. An analysis of these neoplasms is in progress, but two aspects of particular interest might be mentioned. Among the kidney tumors, nephroblastomas, tumors comparable to Wilms' tumors in children, have been observed, and it was found furthermore that such tumors could be induced most readily when weanling rats were started on cycasin. The same type of tu-

mor only rarely occurred in rats placed on a cycasin diet after attaining sexual maturity. The possibility that chemical carcinogenesis at times may be instrumental in the development of Wilms' tumors suggests itself, and several studies are under way to test this by exposing rats at various times during pregnancy to cycasin and observing their offsprings (with Dr. Spatz). The histogenetic background of the nephroblastomas is presently investigated in collaboration with Dr. Gusek.

The intestinal tumors have uniformly occurred in cecum and colon and, as a rule, have been characterized by abundant mucus production whether they were polypoid or broadly infiltrating carcinomas. Studies involving bacterial flora of various segments of the alimentary tract, in particular in relation to their ability to cleave the glycoside, are being started in the hope to learn more about the site of liberation of the aglycone from the glycoside. This type of study, theoretically at least, offers an opportunity to examine one possible cause for about 10 percent of the rats not having developed neoplasms. Cycasin, unless cleaved in the intestinal tract by a β -glucosidase of bacterial origin is excreted unchanged without producing toxic effects. The bacterial flora determines, therefore, whether liberation of the toxin and carcinogen occurs. (Dr. Laqueur.)

Excretion of Ingested Cycasin in Germ-free and Conventional Rats

The glycoside, cycasin, a β -D-glucosyl-oxyazoxymethane, had been shown to be hepatotoxic and carcinogenic in *rats*, *guinea pigs* and *mice*. Investigations *in vivo* by Dr. Matsumoto demonstrated that the aglycone of cycasin produced acute and chronic manifestations of toxicity similar to those described with cycasin. The absence of acute toxic effects following either intraperitoneal injection of cycasin or after oral administration of cycasin in germ free rats, suggested that cycasin was degraded to the aglycone in the alimentary tract of animals. An investigation into the fate of ingested cycasin in germ free and conventional rats was undertaken, and the time required for the disappearance of cycasin in urine and feces after

discontinuation of cycasin feeding was examined.

Germ-free rats excreted 60 to 97% of ingested cycasin in urine and a trace to 32% in feces, while the conventional animals excreted 18 to 32% of ingested cycasin in urine and 0 to 5% in feces. The percent of unaccounted cycasin in germ free rats was 0 to 8% while in conventional animals, it was 65 to 82% of the ingested cycasin. It is assumed at present that the amount of cycasin unaccounted for reflects that part of the ingested cycasin which has been metabolized by the animal. This is supported by other observations in which the severity of the liver injury roughly paralleled the amount of cycasin metabolized.

The residual excretion of cycasin in the first 24 hours after the animals were returned to the basal diet was 0 to 1% in urine of both germ-free and conventional rats. In the feces, germ-free animals excreted 0.3 to 0.7%, while none was found in conventional animals. During the second 24 hours, cycasin was no longer found in the urine and feces of either group.

These results indicated that cycasin was not degraded in the germ-free animal, presumably due to lack of a β -glucosidase of bacterial origin. Therefore, the cycasin ingested by a germ-free rat could be recovered nearly quantitatively in the excreta. The metabolic inability of the germ-free rat to break down cycasin to its aglycone is almost certainly responsible for the absence of toxic manifestations in germ-free animals receiving cycasin. It is of particular significance, therefore, that the recently synthesized aglycone given to us by Dr. Matsumoto is highly toxic in germ-free rats. (Dr. Spatz and Mr. McDaniel [LNE-NIAMD].)

Cycasin and Its Aglycone, Methylazoxymethanol (MAM), Employing Bacteria

The effects of cycasin and MAM on bacteria are being studied in the hope of discovering mechanisms for the hepatotoxicity and carcinogenesis of these compounds in animals. Preliminary experiments have shown that relatively high concentrations (about 20 mM) of MAM are required to inhibit the division of *Salmonella typhimurium*. MAM also acts as a muta-

gen and causes reversion to wild type of a group of histidine requiring *Salmonella mutants* which were also caused to revert by the alkylating agent β -propiolactone. The role of bacteria in deglucosylating cycasin is also being investigated, and an assay for bacterial β -glucosidase using cycasin as a substrate is being developed. (Dr. Smith.)

LABORATORY OF CHEMICAL BIOLOGY

The Laboratory of Chemical Biology continues to have, as its main research goals, problems relating to structure-function relationships in proteins, to the genetic control of biosynthesis, and to three-dimensional structure.

Structure-function Relationships in Proteins

Continuing studies on the amino acid sequences of the extracellular nuclease of *Staphylococcus aureus* have resulted in a nearly complete picture of the covalent structure of this protein. There remains only a series of relatively routine stepwise degradations of small fragments to permit an unambiguous ordering of the amino acid residues. This protein which contains approximately 120 amino acids is simultaneously being examined for its physical and catalytic properties. It has been shown by optical rotary measurements that the native polypeptide contains approximately 50% helix. Crystals of the enzyme have been prepared by Dr. Hazen and Dr. Cotton at MIT, and unit cell dimensions and the nature of the space grouping are now being established. Comparison has been made of the covalent structures of the enzymes from two strains of staph. They appear to be nearly identical. Upon completion of the present chemical and physical work it is planned to place heavy emphasis on the nature of the active center and on the geometric arrangement of the chain in three-dimensions. (Drs. Taniuchi, Anfinsen, Heins, and Suriano.)

Purification of the S-RNA synthetase for tyrosine and alanine from *E. coli* cells is nearly completed. Experiments are now in progress to determine whether these two enzymes of similar function exhibit closely similar chemical and physical properties. These studies will test

the hypothesis that the whole array of synthetases may be variations on the same structural theme. (Dr. Herzig.)

Since the most effective method, in principle, for the study of structural function relationships would be the direct organic chemical modification of amino acid sequence and chain folding, we have continued our collaborative work with Dr. B. Wildi of the Monsanto Chemical Company. Previous studies established that protein amino groups could be blocked by trifluoroacetyl groups. We have now developed a useful reversible blocking agent for carboxyl groups as well, namely N-chloromethyl phthalimide. Bovine pancreatic ribonuclease and the staph nuclease referred to above can be completely masked with this reagent and enzymatic activity can then be regenerated by treatment of the protein derivative at 0° with 1 M piperidine. This situation now permits a systematic study of the trypsin digestion of the blocked protein to cleave arginyl bonds specifically. It is planned to prepare and separate large fragments using such an approach and then to reassemble these fragments with and without prior treatment to shorten or lengthen or otherwise modify individual fragments. Since, as is discussed below, the folding of the polypeptide chain into the specific, catalytically active three-dimensional structure appears to be spontaneous, these studies may eventually permit a partial synthesis of an enzyme of moderate size. (Dr. Wildi, Dr. Goldberger, and Dr. Anfinsen.)

Formation of Tertiary Structure of Proteins from the Primary Sequence

Several further examples of the spontaneous refolding of reduced polypeptide chains in the native form containing the proper pairing of half-cystine residues have been studied. Takaamylase and soy bean trypsin inhibitor can be regenerated from the random uncrosslinked chain in essentially quantitative yields. This process is catalyzed by an enzyme which we have now prepared in pure form from extracts of acetone powders of beef liver microsomes. The purest preparation causes the complete reactivation of a soy bean trypsin inhibitor derivative containing randomly paired half-

cystine residues in less than 5 minutes in a molar ratio of enzyme to protein substrate of less than 1 to 200. It is now fairly clear that the enzyme acts as a protein disulfide isomerase and converts incorrect to correct SS pairs. The purified enzyme is being examined chemically. Its availability now permits a direct study of the mechanism of action. The enzyme is being used as a probe into the thermodynamic stability of various proteins and protein derivatives. It has been shown, for example, that native insulin is rapidly converted to a high molecular weight net work of disulfide bonded chains which precipitate from solution. These experiments suggest that the tertiary structure of insulin is thermodynamically unstable and that the protein may not be synthesized by the simple combination of the two preformed chains through SS bonds. (Dr. Givol, Dr. Goldberger, Dr. Anfinsen, and Dr. De Lorenzo.)

Since the enzyme mentioned catalyzes disulfide interchange, we are using it to examine the equilibrium concentrations formed during the simultaneous oxidation of unlike peptides containing SH groups. Such equilibria, which are very difficult to obtain in uncatalyzed systems, may yield information about the nature of interactions between amino acid side chains which favor the formation of specific disulfide bonds in proteins. (Dr. Fuchs, Dr. De Lorenzo, and Dr. Anfinsen)

Control Mechanisms in Protein Biosynthesis

The genetic control of protein biosynthesis is being studied in two bacterial systems, the synthesis of β -galactosidase in *E. coli* and the formation of enzymes of *Salmonella typhimurium* which are concerned with the formation of histidine. Efforts on the first of these projects have been concerned mainly with the purification of β -galactosidase and the study of its fundamental chemical and physical characteristics. This protein has been shown to have a molecular weight of 540,000 and to be composed of four probably identical subunits, which in turn appears to consist of several different smaller polypeptide units having molecular weights on the order of 40–50,000. Studies are now in progress to establish the degree of difference between these ultimate subunits.

These studies are essential for a proper understanding of the consequences of genetic mutation in the portion of genome which constitutes the so-called Lac operon. This operon is visualized at present by most workers in the field as a series of related structural genes controlling the synthesis of enzymes involved in the uptake and metabolism of β -galactosides. The operon is thought to include a genetic locus which produces a specific repressor substance designed to combine with and inactivate a second locus termed the operator region. Synthesis of messenger RNA corresponding to the structural genes in the operon can only occur when certain inducers are added to the bacterial system that can presumably combine with the repressor and thus free the operator to initiate transcription. It had not been established whether the operator is part of the first structural gene in the series or a completely separate locus not connected with poly-peptide sequence determination. We have now shown fairly convincingly that the β -galactosidases formed by strains of cells both containing, and lacking, an effective operator region, are chemically identical. Galactosidases from cells with an intact operative region and from cells possessing a large deletion in this region have been found to show the same physical properties and to exhibit identical catalytic activities. Their covalent structures also are identical on the basis of amino acid analysis, peptide mapping, and the variety of other chemical treatment.

We are now preparing galactosidase from a number of other genetic strains of *E. coli* and propose to examine the consequences of mutations of various kinds in the structural genes themselves. These mutants will include the so-called polarity mutants and their revertants.

The second bacterial system under study, namely that for histidine biosynthesis in *Salmonella*, has been carefully characterized with respect to the time of appearance of various enzymes in the histidine operon after derepression of the synthetic sequence. It has been shown that the changes in specific activities after derepression, of five of the ten enzymes in the operon series follow a definite pattern: the enzymes encoded into the operon nearest the

operator region are the first to show a rise. Those enzymes encoded progressively farther from the operator region do not begin to show an increase in specific activity until considerably later. The large body of data accumulated has been analyzed by computer methods and the exact time of appearance of a rise in activity for each enzyme has thus been determined with some precision. Pulse-labelling experiments are now being initiated in an effort to examine the question of whether a single large messenger RNA molecule is synthesized for the entire operon or whether the series of individual messenger RNA substances are formed and released for each of the individual enzymes involved. The basis for the sequentiality may be either a sequential formation and degradation of ten separate messenger-RNA molecules or, alternatively, the formation and non-formation of a single huge messenger-RNA molecule that can be "read" by the protein synthesizing system starting only at one end and proceeding only in one direction.

These studies are also now concerned with the biosynthesis of enzymes in the histidine operon in strains containing a large deletion which eliminates information for all but the first or second gene at each end of the operon. An analysis of the time characteristics mentioned above should give some information about whether deletion mutants are actually missing a stretch of DNA or whether the apparently deleted region indicated by genetic tests could conceivably be a replacement of preexisting DNA with "nonsense" DNA which is not translated in the polypeptide structure. (Dr. G. Craven and Dr. E. Steers.)

Proteins and Human Heredity Disease

As part of a continued study on the relationship between protein sequence and conformation extensive statistical correlation was carried out on the basis of data from the large literature on mutational and species variations in protein structure. The results confirm the hypothesis that changes in amino acid sequence are only permissible when they permit the formation of the same three dimensional structure.

In preparation for planned chemical study, the genetics of congenital erythropoietic porphyria was analyzed from all of the existing pedigrees in the literature. All data were found to be compatible with the autosomal recessive mode of inheritance. (Dr. Epstein.)

LABORATORY OF BIOCHEMICAL PHARMACOLOGY

Control Mechanisms for Mammalian Enzymes

The work in this Laboratory has shown for the first time that enzyme levels in animal tissues are controlled by changes in *both* enzyme synthesis and enzyme degradation. For example, glucocorticoid administration causes an increase in tryptophan pyrrolase levels by increasing the rate of synthesis, while tryptophan administration causes a comparable increase in tryptophan pyrrolase levels by inhibiting breakdown of the enzyme. Evidence for these conclusions has been obtained by a combination of kinetic, isotopic, and immunologic technics. Subsequent studies on the stability of tryptophan pyrrolase have demonstrated that the process of enzyme degradation in the intact animal or tissue slices is very complex and cannot be exactly reproduced in broken cell preparations or in metabolically-inhibited tissues.

Similar kinetic studies on the increase in enzyme levels after hormonal administration were carried out with other enzymes. Some gave rapid responses, others slow responses. Careful evaluation, however, revealed that this did not represent specificity of response, but rather reflected differences in the half-lives of the enzymes concerned, i.e., proteins that turn over rapidly show a faster response. This is of considerable general pharmacologic importance. (R. Schimke, C. Berlin, L. Grossbard.)

Another enzymatic system that offers promise for studies of enzyme control mechanisms is *hexokinase*. Four separate types of hexokinase can be found in rat tissue. Each type has different kinetic, electrophoretic, and stability properties, as well as different tissue distributions. (H. Katzen and R. Schimke.)

Microbial Physiology

ACTINOMYCIN ACTION IN *E. coli*. Because the drug, actinomycin, inhibits the synthesis of RNA, it has been of great value in studies of protein synthesis, RNA metabolism, and metabolic control mechanisms. This drug could not be used in studies with *E. coli*, because this organism is normally impermeable, and therefore insensitive, to actinomycin. It now has been shown that this permeability barrier can be temporarily removed by briefly treating *E. coli* with the chelating agent, EDTA. The bacteria become permeable not only to actinomycin, but to a variety of compounds (e.g., o-nitrophenyl- β -D-galactoside, carbamylphosphate) which normally cannot enter *E. coli*. This EDTA treatment does not cause any decrease in viability or in the ability to synthesize proteins or nucleic acids. (L. Leive)

The use of the EDTA treatment for sensitizing *E. coli* to actinomycin should be extremely fruitful since so much is known about the genetics and enzymatic processes of *E. coli*. Several problems have already been undertaken. For instance, the degradation of messenger RNA and the assembly of ribosomes from ribosomal RNA and protein is being studied in actinomycin-inhibited *E. coli*. (L. Leive)

Other studies have concerned the effect of actinomycin on the replication of bacteriophage T4 in *E. coli*. Somewhat surprisingly, extremely low concentrations of actinomycin prevent the formation of mature phage, even though this concentration of actinomycin has no effect on RNA, DNA, or protein biosynthesis. These results suggest that these low concentrations of actinomycin inhibit the packaging of the phage DNA rather than the synthesis of messenger. (D. Korn, L. Leive, J. Protass)

BACTERIOPHAGE (LYSOGENIC INDUCTION). Studies with the lysogenic strain, *E. coli* K12 (λ), have shown: (1) Superinfecting phage are excluded from *E. coli* K12 λ only if prophage DNA synthesis can occur. (2) It is known that *E. coli* K12 λ , infected by λ ind-,

cannot be induced; they are "repressed." If the bacteria are first induced such cells rapidly become resistant to this repression by λ ind-. It has now been shown that λ ind- does get into such cells, but is prevented from acting by some intracellular change occurring immediately after induction. (3) In experiments in which lysogenic induction was caused by mitomycin C, uracil has to be present during the induction period to allow prophage DNA replication to occur. In the absence of uracil, prophage loses the ability to replicate, but can still direct the synthesis of the phage-specific exonuclease. (D. Korn, J. Protass)

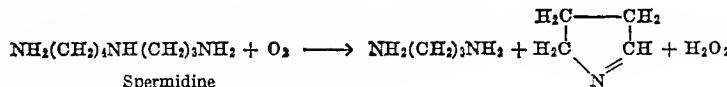
RIBOSOMES. A new method has been developed for the preparation of highly-purified ribosomes from *E. coli*. These ribosomes have considerably higher RNA/protein ratios than the preparations currently in use. (A. Furano) Dye-binding studies have been carried out on ribosomes, and indicate that most of the RNA in ribosomes is in a coil conformation. (A. Furano and D. Bradley)

HFR TRANSFER. Studies have been carried out on the mechanism of chromosome transfer in mating of *E. coli* K12. Preliminary experiments indicate that concomitant DNA synthesis is not required for chromosome transfer. (G. Crowley)

Spermine, Spermidine and Other Amines

This laboratory has been interested for many years in the metabolism of various biologic amines, particularly histamine and various polyamines. During the past year special attention has been directed toward the metabolism of spermine, spermidine, and 1,4-diaminobutane (putrescine). These are naturally-occurring amines, which, in recent years have attracted special interest because of their high affinity for nucleic acids, phospholipids, and other acidic components of the cell.

(1) Further studies have been carried out on the *Serratia* enzyme that carried out the following oxidation:



This enzyme, as opposed to other amine oxidases, will not react directly with molecular oxygen, but required the addition of an electron carrier, such as phenazine methosulfate. Acid ammonium sulfate treatment results in dissociation of the enzyme from its cofactor; activity can be restored by addition of flavin adenine dinucleotide. (A. Campello, C. Tabor and H. Tabor).

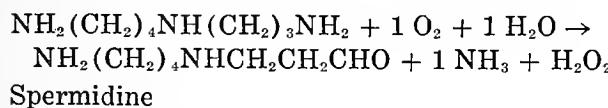
(2) Extensive studies have been carried out on the monoamine oxidase that has been purified 5,000-fold from human plasma. Kinetic studies have demonstrated that the unionized amines are the effective substrate form for this enzyme. Clinical studies have shown that elevated enzyme levels are found in some cases of chronic congestive failure and of chronic liver disease. (C. McEwen)

(3) A monoamine oxidase has been purified 650-fold from rabbit sera, and extensively studied. In contrast to the human serum amine oxidase, the protonated amines are the effective substrates for this enzyme. (C. McEwen)

(4) Active transport systems have been demonstrated in *E. coli* for 1,4-diaminobutane, spermidine, and spermine. (C. Tabor and H. Tabor)

(5) The metabolism of 1,4-diaminobutane and spermidine has been investigated further in *E. coli* and *B. subtilis*. Methods have been developed for the isolation of amino acid-spermidine conjugates from *E. coli*. (C. Tabor, H. Tabor, L. deMeis, D. Savitz)

(6) Studies have been continued with beef plasma amine oxidase that carry out the reaction:



Chemical studies on the reaction product show that, upon heating, the dialdehyde produce is degraded to 1,4-diaminobutane and acrolein. (C. Tabor and H. Tabor)

(7) Derivatives of imidazoleacetaldehyde have been synthesized. This is the postulated product of the diamine oxidase catalyzed oxidation of histamine. (H. Bauer)

Experimental Burns

Experiments with germ-free mice have shown that infection, particularly with *E. coli*, plays an important role in mortality after burns. Because of these observations endotoxin toxicity was studied in burned and in normal mice; endotoxin was about 1,000 times more lethal in burned mice than in normal controls. Mice tolerant to a lethal dose of endotoxin demonstrate significantly lower shock mortality after burns than non-tolerant mice. (K. Markley)

Leprosy

Studies have been continued on improving the growth of *Mycobacterium leprae murium* in cultures of mouse peritoneal macrophages. Best growth was observed when the medium was supplemented with both ferric nitrate and a liver fraction. Under these conditions the generation time of *M. leprae murium* was 7-8.5 days. Studies are in progress to apply these methods to the culture of *M. leprae* (the human strain). Chemotherapeutic studies on murine leprosy *in vivo* have also been continued. (Y. Chang)

Sulfur-containing Compounds

The enzyme glutathione reductase possesses one equivalent of flavin adenine dinucleotide (FAD) per mole and one -SH group appears upon reduction of the molecule with TPNH. It had been postulated that in the oxidized form of the enzyme the sulfur atom of this -SH group is covalently bound to FAD by a TPNH-reducible bond. Such a bond is pictured as being repetitively opened and closed in the course of the catalytic activity of the enzyme. This bond was also thought to be responsible for the inability of FAD to dissociate from the apoenzyme without reduction. Although this may be true, it has now been shown that a second reducible bond, distinct from the one discussed here, is also involved in the binding of the coenzyme. (R. Colman, S. Black and S. Hopper)

Effect of Fasting on Fatty-changes in Mice

The effect of fasting and of exercise was tested on organ weight, water and neutral fat content and histology of various organs. (S. Webster and B. Highman)

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Carbohydrate Metabolism

REACTIONS INVOLVING CARBOHYDRATE POLYMERS. An unusual amino sugar nucleotide, TDP-N-acetyl-3-amino-3,6-dideoxy hexose, was synthesized by extracts of *Xanthomonas campestris*. The acetylated monosaccharide was then isolated in crystalline form, and its structure tentatively determined as N-acetyl-3-amino-3,6-dideoxy-D-galactose. This sugar has been identified as a constituent of a toxic, phenol-soluble lipopolysaccharide obtained from the same organism. (Drs. G. Ashwell and J. Hickman)

Studies have been carried out which, it is anticipated, will provide meaningful insight into the complicated reaction mechanism of deoxy sugar formation. These involve study of the utilization of TDP-glucose specifically labelled with tritium on carbon 3. Degradation studies were carried out on an enzymatic reaction product, L-rhamnose (a 6-deoxy sugar) and the reaction intermediate, TDP-4 keto-6-deoxy glucose. (Dr. O. Gabriel)

A galactose polysaccharide of snails, called galactogen, contains both *D*-galactose and the rare sugar, *L*-galactose. The biosynthesis of this polymer has been demonstrated utilizing UDP-*D*-galactose-C¹⁴ as a galactosyl donor. (Dr. E. Goudsmit)

The mechanism of glycosylation of plasma glycoprotein has been studied. Rat liver microsomes contain the enzyme required to attach N-acetyl neuraminic acid to a variety of glycoprotein acceptors and also contain an endogenous acceptor. Evidence was found that in mucopolysaccharide formation the synthesis of protein is synchronized with formation of the polysaccharide moiety. (Drs. E. Neufeld, P. J. O'Brien and C. C. Levy)

REACTIONS INVOLVING SMALL CARBOHYDRATE MOLECULES. A number of investigations have been concerned with the metabolic control of biosynthetic pathways by feedback inhibition, and some interesting and intricate mechanisms were discovered. These include the following:

1. Certain bacteria contain fucose but not mannose in their polysaccharides, while others do contain mannose, or mannose plus fucose. The biosynthetic pathway leading to these sugars involves formation first of GDP-mannose, then of GDP-fucose. Their synthesis was independently controlled by inhibition of the pyrophosphorylases by these coenzymes. Similarly, CDP-glucose pyrophosphorylase, an enzyme which catalyzes the first step in CDP-paratose biosynthesis, was inhibited by CDP-paratose. (Drs. R. Kornfeld, R. Mayer and V. Ginsburg)

2. In rat liver, formation of UDPG (the glucosyl donor in glycogen biosynthesis) was inhibited by 5'-AMP and by UDPG. (Dr. S. Kornfeld) Also in rat liver, the feedback control of sugar nucleotide biosynthesis was investigated. For example, it was found that UDP-N-acetyl glucosamine inhibited *L*-glutamine D-fructose-6-P transaminase, the first enzyme unique to its biosynthesis. Similarly, the formation of N-acetyl mannosamine by UDP-N-acetyl glucosamine 2-epimerase is inhibited by the end product CMP-U-acetyl neuraminic acid. This feedback inhibition was also demonstrated *in vivo*. UDP-glucose dehydrogenase of liver, peas and cartilage is strongly inhibited by UDP-xylose. This nucleotide is known to be the end product of the UDP-glucose → UDP-glucuronic pathway in peas. (Drs. E. F. Neufeld, P. J. O'Brien, S. Kornfeld and R. Kornfeld)

The general principle emerging from these and many other studies in the literature is that the end product of a biosynthetic pathway controls its rate of synthesis by specific inhibition of an enzyme concerned with an early step in the biosynthesis.

An enzyme fraction from the "high-speed supernatant" of rat testis catalyzed the conversion of glucose-6-phosphate-C¹⁴ to the cyclic compound, inositol. The system requires DPN and Mg⁺⁺. (Dr. F. Eisenberg, Jr.)

A new enzyme activity, dTDP-N-acetyl-D-glucosamine pyrophosphorylase, was shown in extracts of hog gastric mucosa. (Drs. R. Kornfeld, S. Kornfeld and V. Ginsburg)

Intermediary Metabolism of Hydrogen

These investigations are concerned with a rather extreme case of biological specificity—the metabolic behavior of hydrogen atoms whose location in the molecule is slightly different, for example, the metabolic behavior of the two enantiomeric carbinol hydrogens on carbon 1 of fructose-6-phosphate in mammalian liver. For such studies the hydrogens are specifically labeled with tritium (H^3). The initial studies have shown that in mammalian liver tissue the intracellular synthesis of the two distinct H^3 -labeled carbinol hydrogens of fructose-6-phosphate can be effected. One of these is formed on incubation with ribose-1- H^3 , the other on incubation with mannose-1- H^3 . The ribose label eventually appears in position 1 of glucose-6-phosphate whereas mannose-1- H^3 gives rise to fructose-6-phosphate so labeled that on isomerization to glucose-6-phosphate the H^3 appears as triton or in position 2 of glucose-6-phosphate. (Drs. B. Bloom and J. Millet)

Hormones

An investigation is under way to study, at a molecular level, the mechanisms by which hormones regulate morphological and biochemical differentiation and growth of mammalian tissues. Twelve-day pregnant mouse mammary gland explants are cultured in a chemically-defined medium using the floating lens paper technique. A combination of three exogenous hormones, insulin, prolactin and hydrocortisone, was most effective in stimulating secretion of proteinaceous material, in casein biosynthesis and in stimulation of P^{32} uptake into ribosome-associated RNA. Proliferation of epithelial cells was about as good in the absence of hydrocortisone. Further analysis of the effect of individual hormones on this *in vitro* differentiation system is under way. (Drs. Y. Topper, W. Juergens and F. Stockdale)

A purified enzyme from mammalian liver, glutathione-insulin transhydrogenase, catalyzes the reductive cleavage of insulin with glutathione. Recently it has been found to be active with a number of modified forms of insulin. Further, the enzyme stimulated the rate, but not the extent, of formation of insulin-like protein from reduced precursors during air oxidation. Thus, it is possible that the enzyme is involved in generation of action insulin from its polypeptide chain precursors. The enzyme was also active in accelerating the rate of regeneration of reduced pancreatic RNase during the course of air oxidation. (Drs. F. Tietze and H. M. Katzen)

Nucleic Acids and Other Polynucleotides

STRUCTURE AND FUNCTION OF S-RNA. Periodate oxidation of the ribose moiety of the terminal adenosine residue of S-RNA destroys its acceptor activity. Such S-RNA competitively inhibits the enzymatic aminoacylation of native S-RNA, and the effect is specific. Thus, a periodate treated fraction originally rich in valine activity gave good inhibition of the aminoacylation of valyl-s-RNA but not of tyrosyl-s-RNA and *vice versa*. Partial digestion of S-RNA with snake venom phosphodiesterase also yielded an inhibitory product. In other work, an immunochemical method was devised for purification of S-RNA. (Drs. G. Schwartz, J. Torres and M. Kern)

A new, sulfur-containing nucleotide was discovered in S-RNA from *E. coli*. It is 4-thiouridine phosphate, a material never before found in nature. It has been purified and identified. Work is under way concerning its role in secondary structure, and its route of biosynthesis. (Dr. M. Lipsett)

The antibiotics, mitomycin C and porfiromycin, were found to alkylate DNA, ribosomal RNA and S-RNA. Presumably this is part of their mechanism of biological action. From S-RNA alkylated with mitomycin, both a monoguanyl and diguanyl derivative were obtained. (Drs. M. Lipsett and A. Weissbach)

ENZYMES ACTIVE WITH RNA. A highly purified, potassium-activated phosphodiesterase of *E. coli* was found to be extremely spe-

cific for single-stranded polyribonucleotides. It is exquisitely sensitive to secondary structure, being unable to hydrolyze known helical polymers as (poly A + 2 poly U). The enzyme was used to follow the melting out of certain helix-random coil systems. It is possible that this diesterase is responsible for the breakdown of messenger RNA, especially since it is 40 times more abundant than polynucleotide phosphorylase, and 3 times more abundant than polynucleotide phosphorylase, and 3 times more abundant than ribosomal RNase, in extracts of *E. coli*. A general method for the synthesis of trinucleoside diphosphates with polynucleotide phosphorylase was developed. This is very useful for studies of the genetic code. The sensitivity of both polynucleotide phosphorylase and phosphodiesterase to secondary structure was further established by comparing the degradation of free poly U and polymer bound in the complex (2 moles poly U + one mole 2,6-diaminopurine riboside). (Drs. M. F. Singer, F. Howard, P. Leder and R. Brimacombe)

BIOCHEMISTRY OF LYSOGENY IN *E. coli* K12. Lysogenic cells (*E. coli* K12 λ) can be induced to form vegetative phage with the antibiotic, C¹⁴-porfiromycin, and the λ phage thus produced was found to contain the antibiotic. In other studies, it was found that superinfection of such immune cells with the homologous phage λ results in no synthesis of an early phage enzyme, λ exonuclease. This indicates that repression of the superinfecting phage genome occurs before the stage of early enzyme synthesis, probably at the level of transcription of the DNA to form messenger RNA. The mutant phage λ_{vir} is able to overcome the immunity of *E. coli* K12 λ and produce normal yields of phage in these lysogenic cells. However, the λ_{vir} mutant is still partially repressed in lysogenic cells since its ability to produce the λ -exonuclease is repressed about two-thirds when compared to λ_{vir} infection of nonlysogenic cells. This indicates that sensitivity to repression in lysogenic cells may be partial or complete. (Drs. A. Weissbach and A. Lisi and Mr. W. E. Pricer, Jr.)

Enzyme Induction in a Broken Cell Preparation

Partial success has been achieved in preparing a system of digitonin-lysed, penicillin spheroplasts of high synthetic activity, in which newly synthesized β -galactosidase represents as much as 2-3% of the initial protein. The final preparation is not free of cells or spheroplasts. However, by means of visual and viable counting techniques it has been established that the cell count decreases in the course of incubation. Equally encouraging is the demonstration that *E. coli*, particularly after having been exposed to digitonin, form little or no β -galactosidase under the experimental conditions employed. Thus it is unlikely that the observed enzyme synthesis was due to contamination by intact cells. (Drs. I. G. Leder and J. C. Rabinowitz)

Enzymatic Utilization of Model Compounds

Several diverse enzyme systems are under investigation as part of a continuing study of the reactivity of a variety of chemical groupings in enzyme-catalyzed reactions. As one example, the enzyme responsible for the synthesis of niacin, quinolinate phosphoribosyl transferase, has been isolated in crystalline form. It catalyzes a reaction between quinolinic acid and 5'phosphoribosyl-pyrophosphate to give nicotinic acid mononucleotide, CO₂ and pyrophosphate. Free nicotinic acid is not an intermediate. Current experiments are concerned with the confirmation of exchange reactions between enzyme and substrate which are predicted by kinetic analysis of the process. As another example, two enzyme systems for tartaric acid utilization are being investigated with regard to the enzymes and intermediates which are involved. One of these converts L(+)-tartaric acid to oxalacetic acid, and the other converts meso-tartaric acid successively to dihydroxyfumaric acid and *D*-glyceric acid. (Drs. W. Jakoby, P. Packman and L. Kohn)

Release of Surface Enzymes from *E. coli* by Osmotic Shock

E. coli cells were subjected to osmotic shock by suspension in a hypertonic solution of sucrose with 3 × 10⁻⁴ M EDTA, followed by sus-

pension in cold water or 10^{-3} M MgCl₂. The sudden osmotic transition caused 3% of the cellular protein to be ejected into the medium within one minute. Most of the acid-soluble pool was also lost, and yet the cells remained viable. A special set of proteins was released, including alkaline phosphatase, 5'-nucleotidase, cyclic phosphodiesterase, acid phosphatase and RNase. Histochemical evidence (Bruce Wetzel) and other experiments suggest that these degradative enzymes are at or near the cell surface. These experiments are significant for the following reasons: (1) They indicate that degradative enzymes are somehow segregated in the bacterial cell. (2) They provide for biological study viable *E. coli* lacking these degradative enzymes and with increased permeability. (3) They provide an easy and effective route for purification of certain enzymes. (4) Other systems can then be studied in *E. coli* without interference from phosphatases. (Drs. W. Brockman, N. Nossal and M. N. Lipsett)

LABORATORY OF PHYSICAL BIOLOGY

The past year has been a busy and productive one, with about 50 publications, involving about 30 intramural authors. As in previous years we have enjoyed the stimulation of visiting colleagues from abroad; this year's crop including representatives from Chile, England, Israel, Japan, Mexico and Switzerland.

Present interests of LPB scientists happen to fall rather naturally into three main domains, molecular structure, metabolism, and biological energy transduction. In reporting highlights of these investigations only the names of intramural workers making major contributions are given. The numerous additional collaborators, past and present, including those outside our Laboratory, are credited in the various individual reports.

Molecular Structure

The Laboratory of Physical Biology is engaged in an intensive and diversified investigation of the structures of molecules ranging from simple inorganic compounds to macromolecular polymers, using all the standard spectroscopic tools, a battery of automated devices for recording and converting spectral

data into numerical form suitable for calculating the corresponding molecular parameters, and computer matching with theoretical models. When applied to molecules such as hemoglobin or polypeptides the biomedical connotations of such interests are clear. But it perhaps needs to be emphasized that the physico-chemical parameters of simple molecules, esoteric as such data appear, are of possibly even greater importance in the long run, since this information is more basic and broadly applicable. Further, the types of apparatus needed for studying molecular structure are so complex and the principles of operation are so well concealed in "black boxes" that it may be worthwhile to attempt some slight explanation of the enigmatic initials ORD, NMR, and so on.

When quantized electromagnetic radiation passes through a material, some of its energy may be absorbed providing there is a very precise fit between the kind of energy offered—that is, the wavelength (frequency) of the radiation—and the ability of the absorbing molecule to receive it. Absorbed energy may cause a variety of intramolecular changes: rise of an electron to a higher energy level, change of axis of spin of electron or proton, reduction or increase in rotational or vibrational energy of a bond between atoms—all by quantal amounts. Hence by scanning molecules of one substance with a continuous succession of radiation wavelengths it is possible to plot an absorption spectrum, each peak of which is related to a specific structural property or interaction of the absorbing molecule. Whether or not an absorption peak can in fact be referred back to a specific molecular structure usually depends on a laborious comparison of spectra from a large number of related compounds together with computational predictions based on thermodynamic and quantum mechanical theory.

In our Laboratory the classical method of infrared spectroscopy, particularly useful for measuring molecular vibrational and rotational energies, has been used in an important series of investigations resulting in a complete "vibrational assignment" for the p-benzoquinone molecule and a number of its derivatives (Becker, Charney). This means that the

strengths of the two C=O bonds, the four C—H bonds, the four C—C bonds and the two C=C bonds are precisely known and their interactions with each other explained. By the same methods the electronic distribution patterns in germanium and tin hydrides, and trihalides of boron, have been investigated, the data being used to clarify the theory of force fields within these molecules (Levin).

Other studies have been carried out using ultraviolet radiation not in simple absorption spectroscopy but in the more powerful techniques of optical rotatory dispersion and circular dichroism. These methods are particularly adapted to studying "optically active" compounds (those with mirror-image molecular asymmetry), which are often of special biomedical importance because one isomer may be extremely active physiologically, the other completely inactive. The former method (ORD) measures the degree of rotation of plane-polarized light as a function of wavelength; the latter (CD) depends on preferential absorption of right or left circularly polarized light. Both methods yield information about the spatial configuration of atoms in molecules. They have been used in studying the stereochemistry of morphine and codeine derivatives (Weiss). Another of our particular interests has been the class of conjugated cyclic dienes. The basic problem has been to relate the two possible configurations of the structure RC=C C=CR (that is, the "cisoid" case when the molecule is bent at the central C—C bond into a V shape, versus the quasi-linear "transoid" arrangement when one "arm" is up, the other down) to whether the molecular skewing is right or left-handed. Study of numerous representative dienes has confirmed the relation discovered last year that sign of rotational effect is related to handedness (Weiss, Ziffer). A comprehensive analysis of the intensity of these effects by molecular orbital theory has also been completed (Charney).

Two other relatively new spectroscopic methods, employing magnetic resonance, are being widely used in our Laboratory. These methods involve subjecting a solution of the molecules in question to a strong magnetic field, which "freezes" that small fraction of

the total molecular population that contains unpaired electrons or protons whose spin axes happen to be parallel to the lines of magnetic force. When, now, radiation is passed through the molecular aggregation at right angles to the magnetic field, some of the oriented particles may absorb energy of specific (resonant) frequency (wavelength) and "flip" with reference to their spin axes. The exciting radiation for such electron spin resonance (ESR) is in the microwave region, and for proton or nuclear spin resonance (NMR) is in the short-wave radio band. ESR has been used to define the configuration of the unpaired electrons and to account for paramagnetic properties of certain halogen compounds of Cr, Va, Mo, W and Co (Kon). NMR was used in a study of binding of copper by nucleotides (Becker); in an extensive continuing study of DDT analogs and derivatives, with a possibility in view of associating the toxicity with the lability of a particular aliphatic proton (Sharpless); and to elucidate the structure of a series of important carcinogens, the benzanthracenes (Ziffer).

The technique of aligning linear molecules with large dipole moments in a strong electrical potential and then looking at them with polarized ultraviolet light, (Kerr technique) is valuable for determining the size and configuration of polymers. Work of this sort is going forward on polypeptides (Yamaoka). In another unit of our Laboratory evidence is accumulating that the changes in secondary structure of the hemoglobin molecule, brought about by a seemingly minor chemical change in one of the four polypeptide chains of the globin moiety, not only cause the pathological "sickling" of the erythrocyte but actually favor the polymerization of the quasi-tetrahedral hemoglobin units into a beaded linear molecular filament. Indirect evidence for such aggregation from fluorescence microscopy reported last year has now been supplemented by showing that the sickled cells orient in a magnetic field; and by electron microscopy of the extracted hemoglobin molecular filaments (Murayama). Configurational changes brought about by complexing mercury with hemoglobin have also been studied (Resnik).

The most important source of information about crystal structure of macromolecules is

usually X-ray diffraction, but when this fails, information can sometimes be deduced from electron microscopy. Catalase, for example, a heme protein of 225,000 molecular weight, was shown as early as 1937 to crystallize in needle, rectangular plate and quadrilateral prism habits, but resisted efforts to derive its crystal structure by X-ray diffraction analysis. In our Laboratory two additional crystal habits (approximating the regular octahedron and the hexagonal prism) have been prepared, the needle habit has been shown to be actually clusters of long narrow plates, and crystals of any of the four valid habits have been dissolved and recrystallized in all of the other habits by salt concentration control (Labaw).

Metabolism

At the direct biochemical level a comprehensive study of nutrition and growth in the green flagellated protozoan *Euglena* has been completed, including development of a defined synthetic culture medium, elemental and gross chemical analyses and identification of several conventional enzymatic pathways of intermediary metabolism (Kempner, Miller). In other work on Protozoa, four protozoan blood parasites were found to differ specifically in their abilities to synthesize fatty acids from defined substrates (Greenblatt). An interesting new approach to bacterial metabolism is suggested by the discovery that rates of utilization of various substrates and the effects of certain inhibitors during the log phase of growth in *E. coli* are measurable in terms of the amount of electric current produced by the cells (Allen).

A study is also underway of protein synthesis during insect development. The particular interest here is that many of the structures of the immature (larval) stage are completely and rapidly made over during the transformation into the adult stage. In this study differences have been found between the stabilities of larval and adult aldolases, an amino acid previously unknown in nature (L,d methionine sulfoxide) has been discovered, the turnover and incorporation rates of lysine and alanine have been measured and the changes in titer of free amino acids, peptides and proteins in the blood during development have been deter-

mined (Levenbook, Ojeda, Chen) In another investigation major enzymatic changes in the blood plasma of rats were found in response to severe stress by low barometric pressure. It was shown that repeated exercise at atmospheric pressure ameliorated some the damage from subsequent exposure to low pressure (Altland).

In the anatomically simple freshwater animal *Hydra* the conditions under which body cells transform into germ cells look more and more like a complex of quantitative relations rather than the various simple triggers that have been designated in certain other laboratories. For example, the sex-inducing action of a rise in ambient temperature was shown to be quantitatively dependent on both initial and final temperature, and to be independent of the chemical composition of the microenvironment, nutritional state and illuminational regimen (Park, Ortmeyer).

Biological Energy Transduction

The title of this section is an attempt to characterize the class of biophysical processes in which one type of energy is converted into another. In the overall view it is easy to see that light energy is converted eventually to chemical energy in photosynthesis, and that a process descriptively the reverse occurs in bioluminescence. Similarly, chemical energy results in mechanical work in the shortening muscle and in the active transport of cellular water. But as the intimate details of these transductions are investigated, more and more subtle intercalated steps are revealed and it is doubtful that any single instance is yet adequately understood. Our Laboratory is engaged in a broad attack on these problems, including both steady-state situations and those that might be called biological triggers.

Perhaps the best known and longest studied biological transducer is striated muscle. Electron microscopy has shown that the muscle cell contains a precisely oriented array of interdigitating protein filaments running parallel to the long axis of the cell. The two present main theories of muscular shortening provide, respectively that these filaments shorten by intramolecular folding and that they slide to-

gether. Delicate measurements in our Laboratory show that the fine filaments do not change length during shortening of the muscle cell, thus favoring the second view (Podolsky). Calcium ion is thought to be the immediate trigger of excitation, and this view has been strengthened by localization of intracellular sites of calcium storage by electron microscopy (Franzini-Armstrong), by determining the effective concentration range of calcium for excitation (Hellam) and by evidence that the calcium is liberated intracellularly by depolarization of the internal membrane system (sarcoplasmic reticulum) which is probably electrically coupled to the outer cell membrane (Costantin).

The process by which light energy absorbed in the retina is converted to an electrical signal in optic nerves is under intense investigation in many laboratories. In LPB certain steps have been studied in the retina of the squid (which is particularly favorable for the recording of the minute electrical currents generated by the absorbed light because the receptor cells connect directly with single unbranched nerve fibers) and it has been possible to show that the brief pulses of current that result when photons are absorbed do not correspond kinetically to the rates of transformation of any of the known photochemical intermediates. This suggests that many steps intervene between photon absorption and the eventual change in photoreceptor cell membrane permeability, a conclusion supported also by theoretical analysis of the necessary thermodynamic relations between magnitude of membrane current and quantum absorption (Hagins). The sophisticated computerized "shot noise" analysis used to sort periodic electrical signals out of random fluctuations in membrane current is also being applied to photo emission from luminous bacteria and glowing firefly lanterns (Hagins, Hanson).

The transfer of light energy absorbed by chlorophyll molecules, present in an oriented "liquid crystal" state within the layers of the chloroplasts of green plants, to the site where it is used in the basic photosynthetic reaction, has been studied by measuring polarized fluorescence under the microscope. The results point to specific orientation of the pigment

molecules, the pigment itself being a form of chlorophyll absorbing in the far red wavelengths (Olson, Jennings).

It will be apparent that the leit-motiv in all this transduction work is membrane structure, cellular or subcellular. Light-absorbing molecules are oriented on membranes in the outer rod segment and in the chloroplast; changes in membrane permeability accompany the passage of materials into and out of the cell, the generation of the propagated potential of the nerve impulse, the spread of excitation within muscle cell, and presumably in the transfer of nervous excitation to the photocyte.

In our Laboratory, membrane structure is being observed directly by electron microscopy (Hagins, Tice, Greenblatt, Peterson) and "liquid" membranes of strong base ion exchangers are being studied intensively in regard to the physico-chemical carrier mechanism of ion exchange (Sollner, Shean). At the same time collodion membranes of controlled porosity are being used in quantitative analysis of nonelectrolyte and solvent transfer (Gabbay). Another approach is by way of monomolecular films at interfaces. By methods of surface chemistry the complex of forces involved in the specificity and integrity of certain such films have been worked out, binding of charges has been distinguished from electrostatic association of electrolytes with the monolayer and the effects of certain surface interactions of steroid hormones have been systematically elucidated (Gershfeld, Pak). Still another approach is the finding that similarity in ionic radii between arsenate and phosphate allows the former to substitute for the latter in biosynthesis of protozoan cell membrane to give a new lipid species, arsenolipids (Sharpless). Such isomorphic replacement, with presumably parallel permeability changes, may possibly account for the toxicity of arsenic.

This account of LPB activity reaches full circle with the interesting application of nuclear magnetic resonance spectroscopy to intact cells of the protozoan Nocardia. NMR is a probe in which signal profile is sensitive to molecular disorder or asymmetry. Using as a control the signal characteristic of disoriented lipids when the cell became leaky due to damage by Ca and Mg ion or by heat. Whether

NMR spectroscopy will prove a useful tool for studying intact cell membrane behavior remains to be seen, but these preliminary results seem promising.

In conclusion one is very tempted to succumb to the invitation of the Director, NIH, to speculate about the future, particularly in view of the present prevalence of Planning in the air. However, if the history of this elderly Laboratory teaches anything it is that no matter what the original intent in bringing scientists together or to what professional category the scientists nominally belong, no "program" remains stable for very long. If left free to explore, and if not hedged in by artificial organizational boundaries, independent spirits will form a constantly shifting pattern of alliances which defies any tidy system of planning, reporting or prediction. Such uncertainties, however, are more than compensated by the fruitfulness of the interactions in terms of germinating new departures, introducing concepts and techniques from one field into another, and efficient use of available resources. Furthermore, such free collaboration tends to promote an idea-oriented philosophy with emphasis on problem-solving, rather than a tool-oriented philosophy with emphasis on justifying the existence or acquisition of proprietary hardware. We feel, therefore, that aside from the minor amount of judicious and unobtrusive steering that is compatible with research freedom, it is better, and more realistic, to simply take pride in being part of a productive companionship, be thankful for the opportunity to do basic research, and await with interest the next unpredicted shift in the harvest.

LABORATORY OF NUTRITION AND ENDOCRINOLOGY

Guinea Pig Nutrition

In continuing studies on the quantitative nutritional requirements of the guinea pig, the need for methionine was found to be 0.58% of the diet. When adequate cystine (0.24%) was present, however, the requirement for methionine fell to 0.22%. Thus cystine can replace over one-half of the methionine requirement of the guinea pig. (Dr. M. E. Reid)

Metabolism and Function of Fat Soluble Vitamins and Related Substances

Analyses of 140 normal adult serums from NIH employees revealed an average concentration for total tocopherols of 1.06 mg/100 ml, with a range of 0.5-2.0. This contrasted with an average value of 0.76 mg/100 ml, range 0.2-1.5, for 125 serums from villagers in East Pakistan. Re-evaluation of 40 NIH serums by a more precise method showed that α -tocopherol comprised 85-90% of the total tocopherols with γ -tocopherol accounting for the remainder.

Further studies of nutritional factors affecting testis function in rats showed that in the absence of dietary polyunsaturated fat a deficiency of vitamin E does not affect the testis for at least 30 weeks (damage ensues in 18 weeks when polyunsaturated fat is fed). These studies also revealed marked differences from the classical symptoms of essential fatty acid deficiency, in that rats deprived of linoleic acid grew well for 26 weeks and did not have testicular damage even after 34 weeks. The discrepancy is thought to be due to improved dietary formulation assuring adequate tissue saturation of all fat vitamins.

Investigation of the reported reversibility of vitamin E-deficiency testis damage in the hamster has shown that about one-half of the animals had normal restoration of testicular tissue while one-half did not. The difference is thought to be due to the extent of vitamin E depletion prior to resupplementation with α -tocopherol. Even though all hamsters were depleted for similar long periods, there is an apparently marked difference in the retention of trace amounts of tocopherol by various animals. (Dr. J. G. Bieri.)

Metabolic Adaptation to Protein Deprivation

When adult male rats were depleted of protein and oxidative phosphorylation of the liver using succinate as substrate was studied, no difference from control rats was found throughout the depletion period. However, after 2 days of protein repletion, oxidative phosphorylation fell to a very low level and then returned rapidly to a normal level thereafter. The low level after 2 days coincident with a rapid infil-

tration of lipid into the liver. When methionine or cystine was included in the protein-free ration during the depletion, oxidative phosphorylation was considerably lower than in controls throughout the depletion period. It had been shown earlier that inclusion of methionine or cystine in the diet during protein depletion produced very fatty livers. Thus there appears to be somewhat of an inverse relationship between accumulation of fat by the liver under these conditions and capacity for oxidative phosphorylation.

The ratio of succinate oxidation in an actively phosphorylating system (intact mitochondria) to that in a non-phosphorylating system in which mitochondria were disrupted by calcium addition was studied in rats depleted of protein, to investigate one parameter of mitochondrial membrane permeability. This ratio in protein-depleted rats was more than double that in control rats. This effect cannot be attributed to an indirect effect of any one of the components added to the *in vitro* system, such as hexokinase, AMP, glucose, KF, Mg⁺² of glucose-6-phosphate; the effect was the same whether these components were present individually or in various combinations. Thus it appears that the permeability of the mitochondrial membrane to succinate is considerably increased by protein depletion. That the effect is not due to uncoupling of oxidative phosphorylation in the intact mitochondria is borne out by the fact that phosphorylation is normal with respect to the amount of oxygen taken up.

Cytochrome oxidase activity is reduced to 15% of normal by protein depletion. However, cytochrome oxidase concentration (cytochromes a + a₃) as measured spectrometrically falls to only 50% of normal. Thus it appears that while a fairly high concentration of the enzyme is still present in protein deficiency, its activity is not nearly up to par. This discrepancy between activity and concentration can possibly be explained by a loss of mitochondrial phospholipid which occurs in protein deficiency. It has been shown by others that phospholipid is necessary for cytochrome oxidase activity. However, its presence or absence would not affect the spectrophotometric analysis for cytochrome oxidase.

Cytochrome a + a₃, b, c₁, and c concentrations (assayed spectrophotometrically) are all lost to about the same extent during protein depletion (40–50% of normal). After protein repletion it takes at least 8–10 days for the concentrations of these enzymes to return to normal. (Dr. J. N. Williams, JR.)

Lipid Metabolism and Purine-Pyrimidine Balance

Work has continued toward an elucidation of the mechanism by which dietary orotic acid induces accumulation of triglycerides in livers of rats. Earlier work has established that lipid accumulation results from a severe inhibition in hepatic lipoprotein secretion. Furthermore, the inhibition of lipoprotein secretion is preceded by an elevation in hepatic uridine nucleotide concentration, depression in adenine, guanine and pyridine nucleotide concentrations, and an inhibition in pyridine nucleotide biosynthesis. All of these effects of dietary orotic acid are prevented and reversed by small amounts of dietary adenine.

More recent findings are summarized here-with. Livers were examined histologically and with the electron microscope just prior to and during fat accumulation produced by orotic acid feeding. No abnormalities are seen until the third day, at which time small vesicles, presumably containing fat, appear between the double membranes of the endoplasmic reticulum. As the fatty liver develops, these vesicles increase in size and apparently coalesce, leading to the appearance of many round, lipid-filled structures throughout the ergastoplasm of the cell. (Drs. H. Windmueller and Y. Tanaka)

The source of the accumulating liver fat was studied by examining its fatty acid composition. In rats with a high caloric intake, the composition was characteristic for newly-synthesized fat. When caloric intake was restricted, the composition was characteristic for adipose tissue fat, indicating that the lipid was mobilized from peripheral organs before accumulating in the liver.

Purine biosynthesis *in vivo* was measured to explain the orotic acid-induced depression of purine nucleotides. The incorporation of for-

mate-¹⁴C and glycerine-2-¹⁴C into the acid-soluble adenine and guanine of liver was stimulated 3 to 10-fold by orotic acid. A comparable increase was found for the incorporation of these precursors into nucleic acid purines of liver. Depressed steady-state concentrations of purine nucleotides in orotic acid-fed rats are, therefore, the result of accelerated catabolism or utilization and not due to an inhibition of synthesis.

By measuring the development of hyperlipemia following an intravenous injection of Triton WR-1339, the onset of inhibited hepatic lipoprotein secretion in the rat was found to occur after 3 days of feeding orotic acid. Thus, inhibited lipoprotein secretion coincides with hepatic accumulation although both are preceded by progressively diminishing steady-state concentrations of lipoprotein in the circulation.

Chronic fatty liver and depressed plasma lipoprotein concentrations were found in rats maintained for 19 months on a purified diet containing 1% orotic acid. In addition to being fatty, livers of orotic acid-fed rats showed adenomatous hyperplasias, fibroadenomas and bile duct proliferation. There was no evidence of cirrhosis (in collaboration with Dr. G. Laqueur). After 19 months of orotic acid feeding, a dietary supplement of 0.25% adenine sulfate restored liver and plasma lipid concentrations to nearly normal levels in 14 days but did not reverse the other lesions. (Dr. H. G. Windmueller)

Folic Acid

Several of the forms of folic acid have been demonstrated to be co-factors in enzyme systems involving the synthesis of thymidine and methionine. However, the significance of the preponderance of the more complex forms of folic acid as they exist in various tissue and microorganisms has as yet not been elucidated. Our studies in the past year have disclosed that the major component (approximately 75%) of the folate complex in yeast is a highly conjugated form of 5-methyltetrahydrofolic acid. More refined isolation and assay procedures have been developed. (Dr. J. C. Keresztesy and H. A. Bakerman)

Significant progress has been made in the purification and characterization of the properties of folic reductase, the key enzyme in the conversion of folic acid to the metabolically active coenzyme. The enzyme from chicken liver has been purified 8000-fold by a combination of gel filtration and column chromatography. This preparation appears to be essentially homogeneous and thus was used to confirm the previously reported unusually low molecular weight of folic reductase, 23,000. A turnover number of 350 molecules of dihydrofolic acid reduced per minute per molecule of enzyme has been obtained for folic reductase which is in agreement with drug binding studies. The observation that the ratio of activities for the reduction of folic acid vs. dihydrofolic acid is essentially the same with this 8000-fold purified enzyme as compared with the original crude preparations is final proof that a single enzyme carries out the reduction of both forms of folic acid. (Dr. B. T. Kaufman)

Large-scale Laboratory

The increased interest of biochemists throughout NIH in the structure and synthesis of proteins has been reflected in the increased use of the fermentation process equipment. On the average, four 300-liter fermentations are being carried out each full working week. A wide variety of non-pathogenic organisms are grown under particular cultural conditions as requested by the individual investigator. There has been an increased use of the other facilities of this laboratory for the processing of large amounts of natural materials which cannot be done with the usual laboratory equipment. (D. L. Rogerson, Jr. and Dr. J. C. Keresztesy)

Experimental Nutrition

The use of germfree animals continues to offer a sophisticated approach to problems in nutritional experimentation. The role of the microbial flora of the gastrointestinal tract can be shown to be either beneficial or detrimental to the nutritional status of the host animal.

Sufficient amounts of the vitamin, folic acid, were found to be absorbed directly by rats with "normal" bacterial flora to protect such ani-

mals from signs of folic acid deficiency. In contrast, germfree rats develop folic acid deficiency.

The studies on the influence of gastrointestinal flora on amino acid nutrition have shown that the administration of antibiotics only partially reduces the destruction of amino acid when compared with germfreeness. Blacktongue or kwashiorkor producing diets permit much better growth in germfree compared to normal rats.

Experimental dietary liver necrosis studies have been extended. More than one type of liver lesion was found in rats maintained on various deficient diets. In addition to "massive necrosis" other lesions which are, as yet, describable only as atypical were found. The former is more often associated with necrogenic diets lacking vitamin E or selenium. The latter are more often found as the result of the feeding of low protein diets. The addition of large amounts of polyunsaturated fatty acids reduce the time required to produce both types of lesions. While germfree rats are also subject to dietary liver necrosis the development of the usual massive necrosis is delayed in these animals. (Dr. F. S. Daft and E. G. McDaniel.)

Parathyroid Hormone

CHEMISTRY. New methods, based on partition on Sephadex G-100 and chromatography on carboxymethylcellulose, were devised for preparing parathyroid hormone in large quantities. A variety of physical studies, described in previous reports, show that the product is better than 90% pure polypeptide, with a molecular weight of approximately 8500, and 75 amino acids in length. Time digestions of the hormone with exopeptidases have allowed deduction of the amino acid sequences at either end of the molecule. The carboxyl-terminal residue was found to be leucine. Cleavage of the molecule with cyanogen bromide yielded one major polypeptide, 55 amino acids in length from the central portion of the molecule plus smaller peptides, 10 amino acids or less in length, representing the NH₂- and COOH-terminal portions of the molecule respectively. This was in keeping with the findings from exopeptidase digestions that, of the 2 methionines in the molecule, one is located near either end

of the polypeptide chain. The hormone was digested with trypsin and the resulting peptides were separated by the "fingerprinting" technique. Several of the tryptic peptides have been recovered and amino acid analysis completed; the COOH-terminal tryptic peptide was tentatively identified by comparing its amino acid composition with results of carboxypeptidase digestion of the native molecule. (Dr. G. D. Aurbach, in collaboration with Drs. J. T. Potts and L. Sherwood of NHI)

PHYSIOLOGY. Studies were carried out to measure the distribution and half-life of parathyroid hormone in the body and to determine the physiological factors regulating the secretion of the hormone by the parathyroid glands. Using pure bovine parathyroid hormone labeled with ¹³¹I it was found that the half-life of the hormone in the rat is 22 minutes and the hormone is distributed into a space representing 30% of the body weight. Further physiological studies based on the immunoassay of the hormone in plasma and parathyroid gland perfusates confirmed the theory that the rate of secretion of parathyroid hormone is governed by the concentration of calcium in the blood. Acute hypocalcemia stimulates whereas hypercalcemia inhibits secretion of the hormone by the parathyroid glands. This study is the first to prove the theory by direct measurement of hormone. (Drs. G. D. Aurbach and R. Melick, with Drs. L. Sherwood and T. Care of NHI)

STRUCTURE-FUNCTION STUDIES AND MECHANISM OF ACTION OF PARATHYROID HORMONE. Parathyroid hormone stimulates respiration and ion transport in mitochondrial systems; it is possible that these effects are related to the known physiological actions of the hormone in effecting calcium and phosphate transport *in vivo*. The mitochondrial systems afford another test parameter for the hormone and its chemical derivatives; thus structural requirements for activity *in vitro* can be compared with those necessary for activity *in vivo*. The central region of the molecule (approx. 55 amino acids in length, obtained by cyanogen bromide cleavage) retains considerable immunological and *in vitro* activities but is devoid of biological activity as tested *in vivo*. The COOH-terminal region of the polypeptide chain contains a site

necessary for highly energetic immunological reactivity but is relatively unimportant for biological activity. Several amino acids can be removed from the NH₂-terminal portion of the molecule without severely altering any of the activities.

The biochemical mechanism was explored whereby parathyroid hormone stimulates respiration of mitochondria *in vitro*. A sensitive test for this effect was developed by measuring the hormone-induced degradation of ¹⁴C-succinate to ¹⁴CO₂. Analysis of reaction media during hormonal stimulation indicated that parathyroid hormone accelerated the rate at which DPNH, produced from succinate, was reoxidized. It is possible that the parathyroid hormone-induced utilization of DPNH is a manifestation of hormonal activation of a specific ion transport system. (Dr. G. D. Aurbach and Dr. J. T. Potts, NHI.)

In collaboration with Dr. Berton Pressman of the Johnson Research Foundation, it was found that parathyroid hormone *in vitro* induces a net transient potassium influx into mitochondria. This effect is the earliest manifestation of parathyroid hormone yet found and occurs before any change in swelling or respiration can be detected. (Dr. G. D. Aurbach.)

Pituitary Hormones

BOVINE TSH. Highly purified bovine TSH preparations were further separated by electrophoresis on slabs of polyacrylamide. The separated components were then filtered on G-100 Sephadex columns. The components of lower potency were less retarded on G-100 which indicates unfolding or a larger molecular size. Other TSH preparations were fractionated by recycling on G-100 with the separation of components with low and high TSH activity. Amino acid analyses showed that all fractions had a similar composition. This suggests that the differences in potency and various physical properties of the various TSH fractions are not due to differences in primary structure but to secondary and tertiary structure to differences in charge (de-amidation.) An immunoassay procedure for bovine TSH has been developed. (Drs. P. G. Condliffe, M. Mochizuke and G. Martinoli)

MURINE TSH. Studies have continued on TSH in murine pituitary tumors. Previously isolated highly potent preparations of the mouse tumor TSH have been studied by gel electrophoresis using the system described above. The active bands have been identified, separated and their individual potencies have been estimated to be in excess of 100 IU per mg. They differ from the bovine TSH components in that they are more acidic and contain fewer basic amino acids. This is the highest potency ever found for TSH. Gel filtration and density gradient centrifugation of the plasma from pituitary tumor bearing mice show that the hormone in the blood has the same molecular weight and electrophoretic mobility as it does in the tumors. The TSH in blood can be quantitatively recovered and has been purified about 100 times by percolation. (Drs. M. Mochizuki, P. G. Condliffe, and R. W. Bates)

BOVINE GROWTH HORMONE. It was found that, as the pH of dilute solutions of the hormone was lowered from 5 to 2.5, there occurs a transition between two distinct configurations of the molecule. This transition was studied by ultraviolet difference spectroscopy, fluorescence, polarization of fluorescence, viscosity, sedimentation and optical rotatory measurements. The data that were obtained can be interpreted as showing that the growth hormone molecule loses some of its tertiary structure during acidification without there being any change in the helical content of the hormone. If the acidification is carried out in the absence of salt the transition is reversible. In the presence of salts containing various anions, the transition is not reversible and insoluble aggregated forms are produced.

In urea, the same type of transition seen at acid pH can be observed at neutral pH values. At least 8 different configurations of growth hormone were observed in all and the conditions under which these different forms can be transformed one into the other have been established. (Drs. H. Burger, P. G. Condliffe, and H. Edelhoch)

HUMAN GROWTH HORMONE. Human growth hormone was prepared from acetone dried pituitaries by percolation. By using an immunoassay procedure to follow the distribution of growth activity during gel filtration, two dis-

tinct forms of HGH having different molecular weights were demonstrated. Human TSH was obtained during percolation but in order to separate contaminating HGH an additional step is required in the isolation process such as rechromatography on CM-C. (Drs. H. Burger, P. G. Condliffe and R. W. Bates)

PITUITARY HORMONES IN HUMAN PLASMA. The concentration of prolactin in blood in women 2 days post partum was 14 ± 3 mU/ml (range 6 to 29) whereas that in normal controls was less than 5 mU/ml. There was no difference between the two groups in the concentration of growth hormone in blood. This indicates a dichotomy between human prolactin and growth hormone. (Drs. R. W. Bates and J. Roth)

TRANSPLANTABLE PITUITARY TUMORS. It was previously reported that injection of common adrenal steroids into adrenalectomized rats with MtT tumors did not adequately reproduce the action of the adrenal gland in rats with MtT tumors upon organ size. In vitro studies with enlarged adrenals from tumor bearing rats have shown that these adrenals produce two unusual steroids. The same steroids have also been isolated from 1 liter of plasma of rats bearing the MtT. An attempt is now being made to identify the steroids; it is believed that they are 18-OH steroids. (Drs. R. W. Bates and D. Johnson)

Metabolism of Adipose Tissue

GLUCOSE METABOLISM IN ISOLATED FAT CELLS. Phospholipase C, the toxin of *Clostridium perfringens*, is an enzyme that catalyses the hydrolysis of phospholipids (primarily lecithin and sphingomyelin) to diglycerides (or sphingosine) and phosphorylcholine. This enzyme added at low concentrations caused an elevation of glucose uptake by isolated fat cells to the same level observed when fat cells are incubated with insulin. Fatty acid synthesis and other parameters of glucose metabolism which are stimulated by insulin were similarly increased in fat cells treated with phospholipase C. Incorporation of amino acids into protein, thought by some investigators to be a specific action of insulin, was also stimulated by the enzyme to the same degree observed with insulin.

Pyruvate oxidation and incorporation into protein of fat cells were stimulated by both insulin and phospholipase C.

The level of hexokinase in the cell, the first enzyme involved in the metabolism of glucose, was unaffected by insulin or phospholipase C treatment and was found not to be the rate limiting step in glucose utilization by the fat cell. It was also found that complete inhibition by oligomycin of ATP synthesis in fat cell mitochondria did not affect the ability of insulin or phospholipase C to increase glucose utilization by the fat cell. These results suggested that insulin and phospholipase C did not affect the mitochondria, the principal source of energy in this cell.

It was possible to demonstrate, by the use of glucose analogues, that both insulin and phospholipase C increased the rate of glucose transport across the fat cell plasma membrane. These studies also revealed that the transport of glucose by the fat cell is a carrier-mediated process, i.e., transport is not by simple diffusion but is mediated by some process that is stereospecific and which has a selective affinity for different sugars. Phospholipase C neither altered the carrier properties of the transport system nor the ability of insulin to stimulate the transport system. Insulin and phospholipase C did not alter the affinity of the carrier for glucose but only the rate of penetration of the sugar.

It was also found that increasing the concentration of phospholipase C in the incubation medium caused an apparent loss in the response of the fat cell to insulin. This apparent loss in response was actually the result of cell destruction as manifested by the release of soluble glycolytic enzymes to the medium and by the appearance of fat droplets that were released from the cell. Addition of ATP and TPN to the partially or totally disrupted cells completely restored glucose oxidation via the pentose pathway to the levels observed when intact cells were treated with insulin. This is further evidence that hexokinase is not rate limiting for glucose utilization. More importantly, these findings suggest that manifestation of insulin action on glucose utilization requires the presence of an intact cell.

Lysis of the fat cell by phospholipase C action was not unexpected since a number of studies have shown that this enzyme causes the lysis of red cells due to its action on the phospholipids of the red cell membrane. Indeed, this can be taken as evidence that the fat cell contains a similarly constituted plasma membrane. (Drs. M. Rodbell and G. de Cingolani)

EFFECT OF GROWTH HORMONE AND GLUCOCORTICOIDS ON ISOLATED FAT CELLS. Severe ketosis, hyperlipemia and fatty livers develop in well-nourished rats when they are 'totally' pancreatectomized. Earlier studies in this laboratory showed that the above did not develop if the rats were depleted of body fat or if they were deprived of the pituitary or adrenal glands. Subsequent studies showed that the glucocorticoid hormone was the only one needed for the development of ketosis in the hypophysectomized-pancreatectomized rat. However, if the rats had small amounts of insulin remaining in the tissues growth hormone was also required. It was suggested that glucocorticoids and growth hormone produced the above effects by stimulating mobilization of lipid from adipose tissue to the liver.

The effect of growth hormone and dexamethasone on fatty acid release was studied in isolated fat cells prepared from adipose tissue of fasted normal rats. The hormones had very little effect when they were added separately. When the hormones were added together, however, they markedly stimulated the release of both fatty acids and glycerol. The concentration of hormones needed for these effects was 0.016 $\mu\text{g}/\text{ml}$ for dexamethasone and 0.001 to 0.01 $\mu\text{g}/\text{ml}$ for growth hormone; a maximal effect was obtained when the growth hormone concentration was 0.1 $\mu\text{g}/\text{ml}$.

It was also found that addition of very small amounts of insulin, 1 to 10 $\mu\text{U}/\text{ml}$, blocked completely the lipolytic action of growth hormone and dexamethasone. The antilipolytic action of insulin did not require glucose.

The lipolytic action of growth hormone and dexamethasone was slow in onset; no effect was seen until after 1 hour of incubation. This delayed effect was in marked contrast to the rapid effect of the other known lipolytic hormones: epinephrine, ACTH, TSH and glucagon. The slow onset suggested that synthesis of

protein or RNA might be required for the lipolytic action of growth hormone and dexamethasone. It was found that the lipolytic action of growth hormone and dexamethasone was blocked by puromycin (10^{-4}M) and actinomycin-D (10^{-7}M) but not by the aminonucleoside of puromycin (10^{-4}M). Puromycin (10^{-4}M) also blocked the incorporation of labeled histidine, leucine and glycine into protein and actinomycin-D (10^{-7}M) blocked incorporation of labeled uridine into RNA. Actinomycin-D also blocked the small stimulatory effect of growth hormone and dexamethasone on incorporation of labeled histidine and leucine into protein. When actinomycin-D was added two hours after growth hormone and dexamethasone, it did not alter the lipolytic action of these hormones during the subsequent two hours. If the only effect of actinomycin-D, added to 10^{-7}M , is to inhibit RNA synthesis, the above observations indicate that growth hormone and dexamethasone accelerate lipolysis in the fat cell by stimulating RNA synthesis.

The above findings clearly demonstrate that growth hormone and glucocorticoids added together stimulate fatty acid release from fat cells and that this response can be blocked by insulin. In view of the very low hormone concentrations needed for the *in vitro* effects, it is likely that these studies reflect the primary mechanism for mobilizing fatty acids in the living animal; the process is stimulated by an interaction of growth hormone and glucocorticoids and is inhibited by insulin. (Drs. J. N. Fain, V. Kovacev and R. O. Scow)

EFFECT OF HORMONES ON FATTY ACID RELEASE IN VIVO. A technique has been developed in this laboratory for studying the effect of hormones on fatty acid release *in vivo* in the rat. The method consists of collecting blood from the left parametrial fat body via the uterine vein and blood from the saphenous artery. The rate of fatty acid release is determined as the venous-arterial difference in plasma FFA concentration and the rate of blood flow from the fat body. Fatty acid release ($\mu\text{Eq}/(\text{g fat body} \times \text{min})$) averaged 0.005 in fed rats, 0.03 in rats fasted 1 day, 0.06 in rats fasted 2 days, and 0.09 in rats 'totally' pancreatectomized for 1 day. Insulin, 2 units subcutaneously or in-

travenously, lowered blood glucose, blood ketone bodies and FFA release in pancreatectomized rats. In normal rats faster 2 days, however, insulin injected, with or without glucose, lowered blood glucose and ketone bodies but had no effect of FFA release. Infusion of glucagon at 5.0 $\mu\text{g}/\text{min}$, but not at 2.5 g/min , stimulated FFA release in insulin-treated pancreatectomized rats. The results suggest that insulin can stimulate the alpha cells of the pancreas to secrete glucagon and that this effect is not mediated through the blood glucose level.

The effect of growth hormone and dexamethasone on fatty acid release *in vivo* was also studied with this technique. Injection of these hormones increased FFA release within 40 minutes in rats fasted for 1 day. There was no significant increase in blood ketone body concentration until 3 hours after injection. The hormones did not affect the blood glucose concentration. (Drs. V. Kovacev and R. O. Snow)

Experimental Diabetes

PITUITARY DIABETES IN RATS. A transplantable tumor that produces elevated blood levels of growth hormone, ACTH and prolactin induces diabetes in the host rat if 40% or more of the pancreas is removed. Histological examination of the pancreases of these rats by Dr. Lacy of Washington University has shown that β cells are degranulated and vacuolated in those rats which are diabetic. Based on these findings studies have been started in rats with 8.0% of the pancreas removed to determine the role of growth hormone, ACTH and adrenal steroids in the induction of diabetes. The dose-response curves obtained so far indicate that growth hormone and ACTH are of equal importance and act synergistically. The current concept is that the adrenal hormones play only a permissive role. (Drs. R. W. Bates and R. O. Scow)

KETOSIS. Some of the factors affecting ketone body formation in the liver were studied with the collaboration of Dr. Otto Wieland in Munich, Germany. Studies with liver slices confirmed the recent report that addition of calcium ion augmented the formation of ketone bodies from endogenous substrate. Oxidation of exogenous substrate (octanoate), however,

was not increased by the addition of calcium to the medium. It may be that the hepatic lipases which hydrolyze endogenous triglycerides and phospholipids are activated by calcium. This will be investigated further in the near future and may assist in understanding the mechanism of turnover of the complex lipids in the liver slice.

It was found that addition of fructose to liver slices decreased the formation of ketone bodies from octanoate by diverting the octanoate carbon to fatty acid and CO_2 formation. Conversely the addition of octanoate to liver slices metabolizing fructose diverted fructose carbon from fatty acid and CO_2 formation to ketone bodies without decreasing the amount of fructose utilized. The quantitative interrelation between fatty acid oxidation and the metabolism of carbohydrate in the liver awaits kinetic studies on the common intermediates of both systems.

In a collaborative study with Dr. Philip Felts in Munich it was found that increased fatty acid oxidation due to feeding of fat or diabetes mellitus was not associated with decreased levels of citrate in the liver as had been found by others. The level of acyl-Co-A in the liver was directly related to the fat content of the liver and the ketonemia. High levels of acyl CoA were not accompanied by decreased citrate levels as would be expected from the *in vitro* observation of Weiss and Wieland. They found that acyl-CoA is a potent inhibitor of the condensation of acetyl-CoA and oxalacetate to form citrate. Thus, the suggested regulatory role of acyl-CoA on hepatic metabolism was not confirmed *in vivo*. (Dr. S. S. Chernick)

HEPATIC FATTY ACID SYNTHESIS. It is well known that hepatic synthesis of long chain fatty acids (LCFA) is dependent on insulin. Many workers have concluded that the impaired synthesis observed in fasting animals is also the result of insulin lack, secondary to decreased secretion of the hormone. A study was made recently of the effect of 'total' pancreatectomy on fatty acid synthesis in the liver of fasting rats. Synthesis was measured by the amount of labeled acetate incorporated into fatty acids in liver slices and in soluble enzyme preparations of liver. The latter, prepared by homogenization and centrifugation at 100,000

$\times g$ was fortified with ATP, MgCl₂, creatine phosphate and citrate.

It was found that synthesis of LCFA was markedly impaired in liver slices and soluble enzyme preparations from rats fasted for 40 hrs. Synthesis was also reduced in slices from fasted pancreatectomized rats whereas synthesis in soluble enzyme preparations of livers from diabetic rats was only slightly less than that from normal fed rats. Addition of glucose 6-PO₄ accelerated LCFA synthesis in enzyme preparations from all three groups of rats. Maximal synthesis in the presence of glucose 6-PO₄, however, was 3-4 times higher in preparations from fasted diabetic than from fasted normal rats. Administration of dexamethasone *in vivo* increased LCFA synthesis in soluble enzyme preparations of liver, but not in liver slices, of fasted normal rats. At the present time it is not known what factors caused the difference on LCFA synthesis in the soluble enzyme preparations; between fasted normal and fasted pancreatectomized rats. It is conceivable that the difference may be due in part to increased secretion of glucocorticoids in the operated rats since administration of dexamethasone significantly increased LCFA synthesis in the soluble enzyme preparation but not in liver slices. (Drs. R. O. Scow and E. Urgoiti, with Dr. R. O. Brady, NINDB)

LABORATORY OF CHEMISTRY

Section on Carbohydrates

SYNTHESIS OF NUCLEOSIDES. The majority of naturally-occurring nucleosides are typified by the ribonucleosides in which the aglycons are attached *trans* to the C-2' hydroxyl group of the sugar moieties; for convenience, one may call them "*trans* nucleosides." The synthesis of substances of this class is readily effected through the tri-*O*-acylglycofuranosyl halides owing to the fact that the acyl group at C-2 of the sugar derivative participates in the displacement of the halogen atom, ensuring a *trans* attachment of the aglycon. A few *cis* nucleosides have been encountered in nature. Of these, one may cite 5,6-dimethyl-1- α -D-ribofuranosylbenzimidazole (α -ribazole), a moiety of the vitamin B₁₂ molecule, and 9- β -D-arabinofuranosyladenine (spongoadenosine) which oc-

curs in the anaerobic sponge *Cryptotethya crypta*. The remarkable cytotoxicity of this latter substance has attracted considerable attention and aroused interest in other β -D-arabinofuranosyl nucleosides. However, a practical pathway for the chemical synthesis of substances of this class was lacking until Dr. C. P. J. Glaudemans discovered that the readily preparable halide, 2,3,5,-tri-*O*-benzyl-D-arabinofuranosyl chloride, condenses with N-benzoyladenine to give (after removal of masking groups) a substantial yield of spongoadenosine. More recently, Dr. J. D. Stevens showed that tri-*O*-benzyl-D-ribofuranosyl chloride may similarly be used for the synthesis of α -ribazole. These new syntheses, involving the use of non-participating groups in glycosyl halides, appear to offer much promise for the synthesis of *cis* nucleosides and a variety of other difficulty accessible carbohydrate derivatives of biochemical and medical importance. However, in order to make the most effective use of this synthetic approach, we need to know the mechanism whereby the halogen in these glycofuranosyl halides is displaced as well as the stereochemical features of the reaction. Unfortunately the two halides mentioned are amorphous and thus not ideally suited for mechanistic studies. Dr. Glaudemans has, however, succeeded in synthesizing the two anomeric 2-*O*-nitro-3,5-di-*O*-p-nitrobenzoyl-D-arabinofuranosyl chlorides; these are crystalline substances and represent the first known pair of anomeric glycosyl halides which lack a participating group at C-2. Studies of the solvolysis of these halides show that they both give predominantly *cis* products, a finding which suggests that *cis* nucleosides may generally be preparable from glycofuranosyl halides lacking nonparticipating groups regardless of the anomeric configuration of such halides.

Methods for the chemical synthesis of deoxyribonucleosides still leave much to be desired. By way of exploring novel approaches to this problem, Dr. R. K. Ness synthesized 3,5-di-*O*-benzoyl-1,2-dideoxy-D-*erythro*-pentofuranose-1-ene, the first furanose-related glycal. Unfortunately, the tendency of this structure to degrade to furan derivatives has thus far prevented its use for the synthesis of deoxy-nucleosides. Theoretical considerations sug-

gested that the 3,5-di-*O*-*p*-anisoyl analog would prove more stable; Drs. R. K. Ness and M. Haga have succeeded in synthesizing this analog and have found that it is indeed more stable than the di-*O*-benzoyl derivative.

SYNTHESIS OF SUBSTANCES RELATED TO CERTAIN MUCOPROTEINS. It has been postulated that the alkali-labile N-acetyl-D-galactosamine residues in ovine submaxillary gland mucoprotein are attached as esters between C-1 of the aminosugar and the nonpeptide-bonded carboxyl groups of aspartic and glutamic acids. For this reason it is of interest to examine the properties of the C-1 esters of the N-acetyl hexosamines. Dr. R. Harrison has developed a synthesis whereby he has been able to prepare various C-1 esters of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. Dr. T. D. Inch has studied some of the properties of these substances and found the equatorial esters to be markedly more labile than the axial esters.

STUDIES ON THE SYNTHESIS OF STARFISH SIALIC ACID. Dr. L. Warren, while in the Laboratory of Biochemical Pharmacology of NIAMD, discovered a new form of sialic acid in the starfish *Asterias Forbesi* and postulated that it has the structure of N-glycolyl-8-*O*-methylneuraminic acid. In preliminary studies of possible pathways for the synthesis of this substance, two approaches have been pursued. The first approach involved the synthesis of 4-*O*-methyl-D-arabinose, a six-carbon fragment representing carbon atoms 5-9 of the sialic acid. Mr. H. W. Diehl succeeded in synthesizing 4-*O*-methyl-D-arabinose from methyl α -D-arabinofuranoside via 2,3,5-tri-*O*-benzyl-D-arabinofuranose. The synthesis itself is of some general interest as representing a method whereby substituents may be inserted on the carbon atom which normally denotes the oxygen atom of the ring structure of a sugar.

The second approach to the synthesis of starfish sialic acid has involved studies of the selective substitution of reactive groups in N-acetyl-D-mannosamine. Direct benzylation of N-acetyl-D-glucosamine and of N-acetyl-D-galactosamine was shown by Dr. R. Harrison to yield the corresponding benzyl 2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-hexopyranosides. Dr. J. R. Plimmer has now found that applica-

tion of the same process to N-acetyl-D-mannosamine causes epimerization, the product being benzyl 2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-D-glucopyranoside. This difficulty has now been overcome and the synthetic pathway is being pursued further.

HIGHER-CARBON SUGARS. The studies of the higher-carbon sugars and other carbohydrates in the roots of *Primula officinalis* Jacq. were continued by Dr. R. Begbie. In addition to primeverose (6-*O*- β -D-xylosyl-D-glucose), and volemitol (D-glycero-D-manno-heptitol) which had been reported many years ago, and sedoheptulose (D-*altro*-heptulose), D-manno-heptulose, and β -sedoheptitol (D-glycero-D-glucoheptitol) which had been mentioned in the preceding report, the following substances were isolated and identified; glycerol, erythritol, D-xylose, xylitol, myo-inositol, D-glycero- β -D-gluco-heptose (crystalline for the first time), D-glycero-D-manno-heptose, D-allo-heptulose, D-*altro*-3-heptulose, and the same octuloses and nonuloses that had been isolated previously from the avocado, namely, D-glycero-D-manno-octulose, D-glycero-L-galacto-octulose, D-erythro-L-gluco-nonulose, and D-erythro-L-galacto-nonulose. Studies on the carbohydrate constituents of *Sedum* species and of the avocado by Dr. H. H. Sephton, and on Pichi tops (the foliage of *Fabiana imbricata*) with the assistance of Mr. Tracy were continued. A similar study of the roots of *Sedum spectabile* was begun by Dr. H. J. F. Angus.

The phenyl α - and β -sedoheptulosides mentioned in the preceding report have been found by Mr. E. Zissis to be hydrolyzed very readily by acids, even at room temperature. They are extremely sensitive toward alkalies: methanolic sodium methoxide converts them into methyl α -sedoheptuloside (which was synthesized independently for comparison) and dilute aqueous potassium hydroxide converts them very rapidly at room temperature into sedoheptulose and phenol.

MISCELLANEOUS RESEARCHES. Through a transvinylation reaction, 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose has been converted to vinyl 2,3,4,6-tetra-*O*-benzyl-D-glucopyranoside by Dr. C. P. J. Glaudemans. The hydrogenation of

this substance affords ethyl D-glucopyranoside (predominantly the α anomer).

1,5-Anhydro-3,4-di-O-benzoyl-2-O-thiobenzoyl-D-arabinitol, the first thionobenzoate of a carbohydrate derivative, has been synthesized.

Nucleosides containing D-xylofuranose moieties are increasingly being used as intermediates for the preparation of a wide variety of synthetic nucleosides. Mr. H. W. Diehl has markedly improved the preparation of the anomeric D-xylofuranose tetrabenzoates, compounds which are required for the synthesis of D-xylofuranosyl nucleosides.

Dr. H. H. Sephton has demonstrated that vapor phase chromatography may be used for the separation and determination of the hydrolysis products of a methylated xylan as their trimethylsilyl derivatives. This exploratory work constitutes an important improvement in the techniques for the elucidation of polysaccharide structure.

Section on Medicinal Chemistry

EVALUATION OF ANALGESICS. Dose-range-finding experiments were performed on 63 drugs (many from universities and the pharmaceutical industry in the U. S. and abroad). There were 69 complete assays by the Eddy hot-plate method and probit analysis of the data and 50 acute toxicity experiments. During the course of the year's work, it was discovered that the so-called "cleaner" white mouse furnished by the Animal Production Unit since December 1962 is far more sensitive to CNS drugs than the old GP mice. Revised ED₅₀ values for standard analgesics such as morphine, codeine and pethidine will be published. The unit continues to cooperate with and advise the pharmacology unit of the University of Michigan and the Addiction Research Center on addiction studies and serve in an advisory capacity for the Bureau of Narcotics, the Committee on Drug Addiction and Narcotics, FDA and WHO. (E. L. May, and N. B. Eddy).

1-AZONIABICYCLO[3,1,0]HEXANE, A NOVEL RING SYSTEM, AND PRECURSOR OF A β -BENZOMORPHAN. 2-Benzyl-3,4-dibromo-1,3,4-trimethylpiperidine has been isolated as the hydrobromide salt (two isomers from the bromination of

2-benzyl-1,3,4-trimethyl-1,2,5,6-tetrahydropyridine. These were transformed into 6-benzyl-4-bromo-1,4,5-trimethyl-1-azoniabicyclo [3,1,0] hexane perchlorates (two isomers) (I) epimeric at the benzyl substituent. Both structures are based on a *trans*-addition of bromine to the double bond with subsequent backside displacement of the bromine on position 3 by nitrogen. Models indicated that in only one of these is the benzene ring in position to bond with the 4-position of the piperidine ring to give a cyclobenzo-morphan which could be expected to yield β -2,5,9-trimethyl-6,7-benzomorphan. This conversion was realized for this isomer by cleavage of the aziridinium ring with lithium aluminum hydride. With potassium acetate the I isomers were converted to a 2-(hydroxyphenethyl) 3-methylenepyrrolidine (ring contraction) and a 3-hydroxy-4-methylenepiperidine. (E. M. Fry)

4-SUBSTITUTED-8-METHYL-1-OXA-4,8-DIAZASPIRO[4,5]DECANES (SPIROPIPERIDINOXAZOLIDINES. Whereas aromatic aldehydes react with 2-aminoethanol to give Schiff bases to the almost complete exclusion of isomeric oxazolidines, with 1-methyl-4-piperidone the spirooxazolidine derivative predominates. The use of mono-N-alkylated-2-aminoethanols gives exclusively oxazolidines (II), of course. With aromatic aldehydes, the II are sensitive to even traces of acid but can be characterized as picrate salts in dry non-polar solvents. The oxazolidines from N-methyl-4-piperidone are relatively stable in dilute acid. Ethanolamine and N-methyl-N-butyl- and N-(2-hydroxethyl)-, ethanolamines have been used and give the title compounds. Hydrogenation of either the Schiff bases or the oxazolidine isomers with 1 molar equivalent of hydrogen (platinum oxide) gives the corresponding β -amino alcohol. The latter and precursor oxazolidines are of interest as carcinolytic antimicrobial anti-inflammatory and diuretic agents. (J. Harrison Ager).

ALDOLASE INHIBITORS. Treatment of diethyl 2,5-dioxoterephthalate with excess lithium aluminum hydride (LAH) gives trans-2,5-dihydroxy-1,4-dimethylenecyclohexane (III) the structure and configuration of which were assigned on the basis of facile absorption of two molar equivalents of hydrogen (platinum ox-

ide), n.m.r. data and analogy with the course of LAH reduction of other β -keto esters. Hydroxylation of I to 1,2,4/5-tetrahydroxycyclohexanediethanol-1,4 (IV) was achieved ultimately in a reproducible 52% yield with a combination of osmium tetroxide and hydrogen peroxide. In preliminary tests, IV showed activity against P1798 lymphosarcoma. (James G. Murphy).

IMMUNOCHEMISTRY-POLYETHYLENIMINE AND SYNTHESIS OF 4-P-METHOXYPHENYL-1-VINYL-PYRROLIDONE. Viscosity studies on polyethylenimine (PEI) polymers indicate that the discoloration noted for fractionated material is due to an alteration of chain structure. PEI polymer binds copper to the extent of one Cu to 5 nitrogens. The end-point of the binding and a quantitative assay for PEI polymer are determined by a change in the UV spectrum. This copper chelation has apparently caused only a small change in viscosity which probably means that the polymer is highly branched. In a strictly linear polymer, a strong coordinating agent will contract the chain and produce a marked change in viscosity.

The synthesis of 4-p-methoxyphenyl-1-vinyl-pyrrolidone was achieved in 8 steps consisting of (1) Reformatsky cyclization of ω ,4-diacetoxycacetophenone in the presence of ethyl bromoacetate; (2) reduction with 5% Pd-C; (3) diazomethane methylation; (4) reaction with N,N-dimethylethylene diamine; (5) treatment with thionyl chloride; (6) treatment with K-t-butoxide; (7) conversion to the methiodide; (8) Hofmann elimination. Yields are 50% and above in all steps. This monomer will be homo- and co-polymerized after or before O-demethylation. The vinyl group seems sluggish toward catalytic reduction. (T. D. Perrine).

INSTRUMENTATION. In addition to completing a set of 6 high-temperature bank gas chromatographs, in cooperation with the Kontes Glass Company, an addition funnel has been devised that will meter the flow of a liquid reactant into a reaction mixture either at a constant rate or at a constant reaction temperature. (T. D. Perrine).

SYNTHESIS OF BIS-4-(*cis*-2,5-DIMETHYL-1-FORMYL) PIPERAZINYL METHANE. Careful reaction

of *cis*-2,5-dimethylpiperazine with ethyl formate gives 1-formyl-*cis*-2,5-dimethylpiperazine which, on reaction with aqueous formaldehyde in boiling benzene affords the title compound, apparently effective in controlling certain types of mammary cancer. (E. L. May).

6,7-BENZOMORPHAN RESEARCH. Optical resolution of α -5,9,-diethyl-2'-hydroxy-2-methyl-6,7-benzomorphan (V), morphine-like for pain relief in animals and man with lower toxicity and much lower (almost no) drug dependence capacity in monkeys than morphine, gives a *lev*-isomer which is twice as potent as and much less toxic than the racemate. The *dextro*-isomer, although much less effective than its antipode is, nevertheless, codeine-like in analgesic activity. Surprisingly too, its physical dependence capacity in the monkey is intermediate, though nil for V. This indicates antagonism between the antipodes. The *lev*-isomer is being studied. (E. L. May).

α -5,9-Dimethyl-2'-hydroxy-2-trideuteriomethyl-6,7-benzomorphan (VI) has been synthesized by lithium aluminum deuteride reduction of α -2,2'dicarbethoxy-5,9-dimethyl-6,7-benzomorphan in turn prepared from the corresponding *nor* phenolic compound with ethyl chloroformate. The analgesic activity of VI is about $\frac{2}{3}$ that of the N-methyl parent while the pKa is 8.53 compared to 8.38 for the N-CH₃. (T. Ishimaru and E. L. May).

Aluminum bromide cyclization of 3,4-diethyl-2-(*p*-hydroxybenzyl)-2-methyl-1,2,5,6-tetrahydropyridine hydrochloride (VIII) gives, in addition to about equal amounts of α - and β -benzomorphan, 1,5-dimethyl-4-ethyl-7-hydroxy-1,2,3,4,5,10,11,12-octahydrobenzo(g)quinoline as the predominant product (27% yield). Its constitution was determined by n.m.r. mass spectra, by mass, UV, and n.m.r. spectra of its Hofmann elimination product (VIII) and by isolation of 7-methoxy-1-methylnaphthalene after aromatization of VIII. This predominant product, resulting from a shift of the 3,4-double bond to the 3-exo position followed by cyclization is nearly as potent as morphine despite its structural nonconformities. (E. L. May, B. C. Joshi, A. E. Jacobson with H. M. Fales and J. W. Daly).

Similar cyclization of the 3-ethyl-4-methyl compound corresponding to VII gave, in addition to the expected benzomorphans, a by-product identical to one obtained earlier by aqueous HBr cyclization. On the basis of an n.m.r. spectrum this product appears to be not the azaanthracene structure but an indano compound resulting from 5-membered ring closure of the tetrahydropyridine. (J. H. Ager).

Synthesis of 5-butyl-, amyl-, and hexyl-2'-hydroxy-2-methyl-6,7-benzomorphan and α -5,9-dimethyl-2-hexyl-2'-hydroxy-6,7-benzomorphan completes each series from methyl to hexyl. In the 5-alkyl series, C₁, C₅ and C₆ confer potency comparable to morphine; activity is nil for C₂, C₃ and C₄. The N-propyl compound is one of the most potent morphine antagonists known. (B. C. Joshi, C. Chignell and E. L. May).

Replacement of 2'-OH of the benzomorphan nucleus with nitro, amino, chloro or fluoro has a detrimental effect on both analgesic potency and toxicity. During the course of synthesis of the 2'-chloro compound, two isomeric by-products were uncovered in the Stevens rearrangement of 1-p-chlorobenzyl-1,3,4-trimethyl-1,2,5,6-tetrahydropyridinium chloride which on the basis of infrared, n.m.r. and mass spectral data appear to be 4-p-chlorobenzyl-1,3,4-trimethyl-1,2,5,6-tetrahydropyridine and 1,3,3,-trimethyl-2-p-chlorobenzyl-4-methylenepiperidine. (A. E. Jacobson).

A NARCOTINE DERIVATIVE. Reaction of cotarnine, a cleavage product of the abundant opium alkaloid, narcotine, with 3-phenylpropylmagnesium bromide has given a good yield of 1-(3-phenylpropyl)-2-methyl-6,7-methylenedioxy-8-methoxy-1,2,3,4-tetrahydroisoquinoline, codeine-like in analgesic activity and active against a resistant strain of *Staphylococcus aureus*. It also appears to have a diuretic effect in mice. (A. E. Jacobson).

QUININE AND QUINIDINE POLYGALACTURONIDES. Reaction of quinine and quinidine (separately) with polygalacturonic acid (mol. wt. 40,000-80,000) in aqueous methanol, filtration, vacuum evaporation to dryness and exhaustive extraction of the residue with boiling acetone gave in each instance, white, sparingly soluble amorphous powders. These salts were

prepared for Dr. N. R. Shulman for immunologic studies in clinical hematology. (L.J. Sargent).

UNNATURAL NUCLEOSIDES. Unsuccessful attempts to construct the quinazoline-dione nucleoside system include (1) acylation of the glycosyl amine prepared from *o*-cyanoaniline and glucose with ethyl chloroformate led to the corresponding tetra-O-carbethoxy urethane which did not cyclize to the expected nucleoside with sodium ethoxide; (2) ethoxide treatment (for cyclization) of the product obtained in reacting 2,3,4,6-tetra-O-benzyl glycosyl chloride with sodio-*o*-cyanophenylurethane; (3) condensation of the sodio derivative of 2,4-quinazoline dione with acetobromoglucose in dimethylformamide. (L. J. Sargent)

DIHYDROCODEINONE ISOMERS. Separation of the isomers (N closed to the 5 or 7 position) obtained in the alkaline cyclization of 1,7-dibromodihydrocodeinone dihydromethine is achieved more effectively before catalytic removal of the 1-bromo substituent. A Favorskii cyclopropane intermediate which can be attacked by nucleophilic nitrogen at either the 5 or 7 position, is suggested to account for the unexpected cyclization to position 5. (L. J. Sargent and B. C. Joshi).

Section on Metabolites

Batrachotoxin, THE STRONGEST VENOM KNOWN. Further progress has been made on the problem of the structure of batrachotoxin. Three congeners, named batrachotoxinin A, B and C, have been isolated by preparative thin layer chromatography. A potential pyrrole system has been detected in batrachotoxin by the strong color reaction with Ehrlich's reagent and *para*-dimethylaminocinnamaldehyde. Much of the toxic effect appears to be due to a selective cardiotoxicity, to judge from current pharmacological investigations by Dr. Lou Harris at the Sterling-Winthrop Research Institute. NMR and mass spectrophotometric data have now supplied all the information possible. In order to elucidate the structure x-ray crystallography will now be employed. Dr. John W. Daly is currently looking into the cultivation of suitable crystals of the methiodide,

as well as *para*-iodophenylhydrazone derived from batrachotoxin and batrachotoxinin A.

STRUCTURE AND SYNTHESIS OF THE GRAMICIDINS. By countercurrent distribution and redistribution the commercially available peptide antibiotic gramicidin was resolved on a preparative scale. The group of lipophilic peptides contained gramicidin A, B and C, each of which consisted of a pair of congeners, *i.e.*, valine-gramicidin (80–95%) and an isoleucine-gramicidin (5–20%). The sum of aromatic amino acids was always 4, namely 4 Try in gramicidin A, 3 Try+1 Phe in gramicidin B and 3 Try+1 Try in gramicidin C. A more hydrophilic, strongly antibiotic group of peptides, designated gramicidin D, contained 5–6 additional amino acids. Complete amino acid analyses, including time-dependence studies of hydrolyses, were carried out over the entire range of 999 transfers (E. Gross).

Gramicidin A is a *linear N-acylated pentadecapeptide ethanolamide*. Its N-terminal L-valine or L-isoleucine is blocked by a formyl group which is cleaved by mild methanolysis at room temperature and easily identified as formic acid by gas chromatography or as formaldehyde after reduction by the chromotropic acid assay. Reformylation of desformylgramicidin A, *via* O-formylgramicidin A, gave back gramicidin A. Desformylgramicidin A was subjected to ten successive Edman degradations under conditions modified to accommodate the insolubility of its residual peptide fragments in water. The phenylthiohydantoins, obtained by cyclization with trifluoroacetic acid, were assayed by gas chromatography up to step 10. In addition, the residual peptides up to step 10 were hydrolyzed and their amino acids determined quantitatively. Selective N-bromosuccinimide (NBS) cleavage of the bonds following the four tryptophans in gramicidin A releases aminoethanol and the NBS oxidation product of leucyl-tryptophan, but no dioxindolalaninespirolactone fragment expected from a Try-Try sequence. These findings combined with the older observations suggest the sequence: HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L - Val-D-Val-L - Try-D-Leu-L - Try-D-Leu-L - Try-D-Leu-L - Try-D-Leu-L - Try-NH-CH₂-

CH₂-OH for *valine-gramicidin A* and HCO-L-Ileu . . . for *isoleucine-gramicidin A*. Thin layer chromatography, optical rotatory properties in various solvents, molecular weight studies by ultracentrifugation and osmometry, are explicable in terms of easy association of at least two molecules of gramicidin A whose unprecedented alternating sequence of (mostly hydrophobic) L- and D-amino acids permits a characteristic secondary structure dependent on the nature of the solvent (R. Sarges).

Coupling of the octapeptide derivative Z-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-N₃ with the heptapeptide ethanolamide L-(H)Try-D-Leu-L-Try-D-Leu-L-Try-D-Leu-L-Try-NH-CH₂-CH₂-OH, or, more conveniently, of the pentapeptide Z-L-Val(or L-Ileu)-Gly-L-Ala-D-Leu-L-Ala-OH with the decapeptide ethanolamide R-L-Val(or L-Ileu)-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-Try-D-Leu-L-Try-D-Leu-L-Try-NHCH₂CH₂HO, having all the properties of N-carbobenzyloxydesformyl (=Z), desformyl- (R=H), and (R=CHO) valine- or isoleucine-gramicidin (R. Sarges).

The volatile acidic fragment in the hydrolysate of gramicidin B and C was identified as formic acid which, after reduction to formaldehyde, was assayed by the chromotropic acid test. The primary sequence of gramicidin B (C), which in the sample examined consisted of 89% of valine-gramicidin B (C) and 11% of isoleucine-gramicidin B (C), was determined by a 14-step Edman degradation on desformylgramicidin B (C) (obtained from gramicidin B (C) by mild methanolysis) as HCO-L-Val(L-Ileu)-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-L-Try-D-Leu-L-Phe(L-Try)-D-Leu-L-Try-D-Leu-L-Try-NH-CH₂CH₂-OH (R. Sarges).

INTERACTION OF MERCURIC ACETATE WITH THE INDOLE CHROMOPHORE IN TRYPTOPHAN DERIVATIVES AND PROTEINS. Indole reacts with two moles of mercuric acetate to give 2,3-diacetoxy-mercuri-indole. Reaction of indoles with excess mercuric acetate in 50% acetic acid results in a bathochromic shift of the spectrum. The spectral changes are useful in the detection of the indole grouping in the free state and bound as tryptophan in proteins.

Mercurated indoles regenerate the parent indole on reaction with thiols or strong cation exchangers. Mercurated indoles retain the susceptibility of the present indoles to oxidation by NBS, whereas phenols, such as tyrosine, become resistant to oxidation in the presence of excess mercuric acetate. The use of mercuric acetate for the selective oxidation of indoles, in the presence of phenols, with NBS is indicated. Electron-withdrawing groups on both the 2- and 3-positions of indole as well as halide ions prevent the normal spectral shifts seen with mercuric acetate (L. K. Ramachandran).

2-Acetamido- and 2-benzamido-alanyl-glycine and methallylglycine peptides and amides are cleaved by heating at 80° in ethanol in the presence of excess mercuric acetate. The amine component is released in yields up to 30%. The treatment of rufomycin A in similar fashion releases certain amino groups from linkage. The released amino groups belong to 2-amino-hexenoic acid, leucine, alanine and O-NO₂ tyrosine. At pH 5.5 and 80° Ce⁺⁺⁺, La⁺⁺, Hg⁺⁺, CO⁺⁺, and Ca⁺⁺ do not cause any rupture of the peptide bonds in rufomycin A. However, some of these ions catalyze the hydrolysis of about 40% of one bond at lower pHs. In the presence of HBr, HCl, and to a lesser extent HF, at room temperature the peptide bond formed by the amino group of 2-amino-hexenoic acid, in O-methyl-dihydrorufomycin A, was particularly labile (H. Iwasaki).

REACTIVITY OF TRYPTOPHAN IN NATIVE AND INACTIVATED ENZYMES. The extent of oxidation of bound tryptophan, in addition to being dependent upon pH (increasing as the pH is decreased), was also found to be very sensitive to buffer concentration and temperature. α -Chymotrypsin, inactivated by reaction with diisopropylphosphoryl fluoride (DFP), *p*-nitrophenyl acetate or N-tosyl-L-phenylalanine chloromethyl ketone were found to be significantly less reactive toward oxidation by NBS at pH's 5.5, 6.0 and 7.0 than chymotrypsin itself. The zymogen was also slightly more resistant to oxidation at these pH's. Trypsin inactivated by reaction with DFP or with the naturally-occurring polypeptide inhibitor from bovine pancreas was also more resistant to NBS oxidation than native trypsin; the effect of inhibition on tryptophan-oxidizability being

dramatic in the latter case. Trypsin, partially inhibited with synthetic polypeptides of L-glutamic acid and L-leucine or L-tyrosine did not exhibit any difference in the reactivity of its tryptophan from native trypsin. Thus the modes of inhibition of the natural and synthetic inhibitors probably differ. α -Chymotrypsin in the presence of large excesses of the inhibitors, benzoic acid or N-acetyl-DL-phenylalanine, showed no detectable difference in tryptophan oxidizability when compared with α -chymotrypsin in the absence of the inhibitors (T. F. Spande and N. M. Green).

A WATER-SOLUBLE PROTECTING GROUP FOR PEPTIDE SYNTHESIS. For the synthesis of acid-labile peptides, especially for those that contain tryptophan, the water-soluble acetyl-methionyl group provides good protection and is easily removed by cyanogen bromide (E. Gross).

SELECTIVE CLEAVAGE OF PROTEINS BY CYANOGEN BROMIDE. Cyanogen bromide has been used for the selective cleavage of the following proteins: brazil nut allergen, chymotrypsin, carbonic anhydrase, glutamic dehydrogenase, pepsin, trypsin and rhodopsin. Current investigations aim at the cleavage of certain fractions from human globulin, especially from α_2 -globulin (E. Gross, and R. Axen).

CYANOGEN BROMIDE CLEAVAGE OF S-METHYLCYSTEINE PEPTIDES. *Cysteine:* N-acyl derivatives of the lower homologue of methionine, *i.e.*, S-methylcysteine, react also with cyanogen bromide. The peptide bond in N-acylaminoacyl-S-methylcysteine is subject to cleavage by cyanogen bromide. The reaction proceeds by participation of the peptide carbonyl of the amino acid that precedes S-methylcysteine. An oxazolidinium salt is formed as intermediate and easily hydrolyzed to O-(N-acylaminoacyl)-serine, the commonly encountered product of the N \rightarrow O-acyl shift of N-acylserine. Special reaction conditions are required for the hydrolysis of the O-acyl derivative. Model peptides such as N-acetylglucyl-, N-acetylvalyl-, N-acetylprolyl-, and N-acetylleucyl-S-methylcysteine were cleaved in yields of over 60%. The reaction with cyanogen bromide is not restricted to the S-methyl derivative of cysteine. Numerous S-alkyl-N-acyl derivatives of cysteine were

successfully reacted with cyanogen bromide. The general applicability of the cyanogen bromide reaction to S-alkyl-N-acylcysteine offers convenient access to many O-acyl derivatives of serine (E. Gross).

SYNTHESIS AND BIOSYNTHESIS OF DEHYDROBUFOTENINE AND ROLE OF THE ENZYMES INVOLVED. The synthesis of the tricyclic serotonin metabolite in toads, namely, dehydrobufotenine, has been achieved via 5-benzyloxy-6-nitrogramine and five subsequent steps. The conversion of tritiated precursors such as tryptophan, 5-hydroxytryptophan, N-methylserotonin and bufotenine in the toad (*Bufo marinus*) has been investigated. The following enzymes play a significant part in this conversion: aromatic decarboxylase, tryptophan-5-hydroxylase, and a special enzyme system that catalyzes dehydrogenation to the tricyclic dehydrobufotenine. Attempts to characterize and isolate these enzymes are in progress (S. Senoh, J. Daly, and C. Creveling).

MANIPULATION OF THE BIOSYNTHESIS OF COLLAGEN THROUGH FLUOROPROLINES. *cis*- and *trans*-4-Fluoro-L-prolines were prepared stereospecifically by S_N2 displacements of tosyl-oxy groups of natural and allo-N-carbobenzoyl-oxy-O-tosylhydroxy-L-proline methyl esters with potassium fluoride. The pure fluoroprolines were catalytically tritiated to uniformly labeled *cis*- and *trans*-4-fluoro-L-prolines. Both fluoroprolines were incorporated into chick embryo collagen and apparently more so than into normal protein. Only *trans*-fluoroproline bound in procollagen was hydroxylated by proline-4-hydroxylase to tritiated hydroxyproline, which was isolated. The fluoroprolines make possible the study of collagen biosynthesis in chick embryos and raw paw pouches. The manipulation of proline hydroxylase and the competitive incorporation of fluoroproline into procollagen will be studied in its effect on tissue regeneration and wound healing (Y. Fujita).

NEW AMINO ACIDS AND PHARMACODYNAMIC AMINES. A microsomal enzyme system has been studied in considerable detail which is capable of converting a wide variety of phenols to catechols. Among the transformations of special interest are the conversion of N-acetylserotonin to N-acetyl-5,6-dihydroxytryptamine

and the formation of catecholamines from phenolic precursors, such as dopamine from tyramine and (nor)-epinephrine from (nor) synephrine. Metanephrine is hydroxylated to a trihydroxy-compound related in structure to mescaline. This establishes a metabolic interaction of (nor) epinephrine and mescaline. Inhibitor studies have indicated that more than one enzyme is involved in these hydroxylations. The hydroxylation has been shown to occur with replacement of halogen in certain compounds. Some correlations of structure versus activity bearing on the mechanism have been made.

2,3-Dihydrotryptophan has been prepared by two different reductions, one involving the bis-trifluoroacetyl derivative. Tritiated material was demonstrated to be transported into brain, to form 2,3-dihydroindoleacetic acid probably *via* decarboxylation of the intermediate dihydroindolepyruvic acid. This amino acid was not a substrate for aromatic amino acid decarboxylase but along with isotryptophan was a weak inhibitor. N-Acetyl-isotryptophan was a competitive inhibitor of chymotrypsin. 2,3-Dihydrotryptamine was converted *in vivo* to 2,3-dihydroindoleacetic acid probably *via* monoamine oxidase.

In order to facilitate assays of the important enzymes, dopamine- β -oxidase and tryptophan hydroxylase, methods were developed for preparation of 5-tritiotryptophan and β,β -ditri-tyramine. These compounds are now being used to study the distribution and isolation of these enzymes (B. Witkop, J. Daly, Y. Fujita, K. Takase, A. B. Mauger and C. R. Creveling).

METHYLTRANSFERASES. The use of catechol-O-methyltransferase and radioactive S-adenosylmethionine has been developed as a useful tool for studying the formation of a variety of catechols from simple phenols. An enzyme which hydrolyzes S-adenosylmethionine to S-adenosylhomocysteine and methanol has been characterized and has been found to be quite widespread in mammalian tissues. Knowledge of this enzyme's abundance in pituitary gland allowed a chemical classification of tumors of this organ. An enzyme which methylates phenol to anisol has been studied. This is one of the few known cases of methylation of a simple phenol in a mammalian system. The enzyme catechol-O-methyltransferase has been extensively pu-

rified for studies on methylation of catechols (J. Daly, B. Witkop and S. Senoh).

NOVEL HYDROXYAMINO ACIDS. γ -Hydroxy-L-lysine prepared from L-lysine by photochemical chlorination and subsequent reaction with silver acetate was cyclized with nitrosyl chloride to a mixture of 80% *cis*-4-hydroxy-L-piperolic acid and 20% *trans*-4-hydroxy-D-piperolic acid. This established the *threo*-configuration for γ -hydroxy-L-lysine, which was converted to *threo*- γ -hydroxy-L-homoarginine, identical in all respects with the naturally-occurring amino acids from *Lathyrus* seeds (Y. Fujita, B. Witkop, and N. Izumiya).

REARRANGEMENT OF ISOCYANO-PEPTIDES. Dehydration of gramicidin A (a linear N-formylpentadecapeptide ethanolamide) with a large excess (~40 moles) of tosyl chloride in pyridine led to the unstable isocyanide. Comparatively stable isocyanides were obtained by dehydration of N-formyl-DL-valine methyl ester and ethyl N-formyl-DL-valyl-glycinate. By contrast N-formyl-DL-valine amine on dehydration gave α -formylaminovaleronitrile, and N-formyl-DL-valyl-glycine amide gave N-formyl-DL-valine cyanomethylamide via the initially-formed unstable isocyanide by a rearrangement which is pictured as an intramolecular dehydration (F. Sakiyama and B. Witkop).

PROLINE ANALOGS AND THEIR EFFECTS UPON THE BIOSYNTHESIS OF ACTINOMYCINS. Synthetic 3-methylproline has been separated into *cis* and *trans* isomers by several methods, and these isomers have been identified by steric hindrance studies. Derivatives of 3-methyl-4,5-dehydroproline have been synthesized. *cis*- and *trans*-3-Methylproline are both potent inhibitors of actinomycin biosynthesis; 4-methylproline is less inhibitory and is incorporated into the actinomycin peptides in place of proline. Tritiated actinomycin acid IV was prepared from tritiated actinomycin IV which had been produced biosynthetically; its enzymic lactonization was not observed. A novel route was used to prepare 2,3-dihydrotryptophan labeled in the 2- and 3-positions, which is required for metabolic studies (A. B. Mauger and B. Witkop).

REDUCTIVE PHOTOCHEMISTRY OF FREE AND BOUND (AROMATIC) AMINO ACIDS. The three

aromatic amino acids undergo extensive reactions when irradiated with uv-light of varying wave-lengths in the presence of NaBH₄. The reactivity under photoreductive conditions as well as the products formed vary in a characteristic way for each amino acid. Tryptophan, histidine and tyrosine undergo partial reduction of the aromatic portion of the molecule. From the photolysis solution of tryptophan 2,3- and 4,7-dihydro-tryptophan were isolated. N-Acetyl-tryptophanamide is partially cleaved to indolepropionic acid, whereas N-chloro-acetyl-tryptophan yields N-acetyl-tryptophan and β -indolylpyroglutamic acid under photoreductive conditions. Imidazoline and imidazolidine structures have been assigned to the products obtained from histidine and its derivatives. The question of dimerization of the reduced histidine is presently under investigation (B. Witkop, F. Sakiyama, O. Yonemitsu, J. Moore, T. Spande and P. Cerutti).

REDUCTIVE PHOTOCHEMISTRY OF FREE AND BOUND NUCLEOTIDES. In the photo excited state uridine and its phosphorylated derivatives are reduced selectively in the presence of NaBH₄ to their 5,6-dihydro derivatives, whereas none of the other major nucleosides (-tides) are reduced. Dihydouridylic acid and dihydrouridine-5'-phosphate were synthesized on a preparative scale by this new method. The applicability to polynucleotides was demonstrated with poly U, yeast RNA and s-RNA from different sources. The specific loss of recoverable uridylic acid and the formation of dihydouridylic acid have been shown to be a function of exposure to photoreductive conditions. This new method for the synthesis of oligo- and polyneucleotides containing dihydrouridylic acid residues permits the study of the function of these naturally-occurring residues in biogenetics, mechanism of base pairing, selective modification of triplets and mutagenic experiments by reductive flash photolysis (P. Cerutti, K. Ikeda, G. Ballé and B. Witkop).

ELECTROLYTIC CLEAVAGE OF TYROSINE PEPTIDE BONDS AND EFFECTS OF TERTIARY STRUCTURE. The cleavage of tyrosyl-peptide bonds by electrolytic oxidation has been applied to vasoressin and angiotensin, in each case showing total selectivity. In the case of ribonuclease, ox-

idation is incomplete, suggesting that selective attack at surface sites may be feasible. Under the reaction conditions, cystine is partially oxidized to cysteic acid (L. A. Cohen and L. Farber).

MECHANISM OF OXIDATION OF TOCOPHEROL AND UBIQUINONE. The oxidation of tocopherol by peroxides has been shown to proceed by an ionic rather than a radical mechanism. The pathway followed in phenol oxidations, in general, is determined, not by the valence change of the oxidant, but by its potential, in addition to other experimental factors (M. Oxman).

CATALYSTS OF ESTER HYDROLYSIS AS ENZYME MODELS. The anion of N-acetylcycloserine, being an " α -nucleophile," is a potent catalyst for the hydrolysis of *p*-nitrophenyl acetate at neutral pH. Hydrolysis occurs by acetyl transfer, the acetyl group being accepted by the nitrogen atom of the heterocyclic ring (G. Milne).

Section on Analytical Services and Instrumentation

Approximately 10,500 chemical and instrumental analyses were performed for 150 research scientists of NIH and for several scientists in other government agencies. Of this number many were non-routine determinations which required a varying amount of laboratory and literature research. While the total number of analyses performed has declined somewhat over the past several years the work load remains about the same owing to the wider variety of more complex analytical services being rendered. Special assistance was given to many research workers in the solution of non-routine analytical problems (W. C. Alford and H. K. Miller).

Section on Steroids

CHEMICAL STRUCTURE OF TOMATILLIDINE. Wolff-Kishner reduction of tomatillidine afforded deoxotomatillidine (II) and catalytic reduction of the latter gave 5,6-dihydrodeoxotomatillidine (III). Treatment of III with acetic anhydride yielded the O,N-diacyetyl- Δ^{22} -dihydrodeoxo derivative (IV) which by acid hydrolysis was readily converted to 3 β -acetoxy-26-acetyl-

aminocholestane-22-one (V). V was oxidized by chromic acid into 3 β -acetoxybisnorallocholic acid. These conversions established the structure of deoxotomatillidine (II) as 25(R)-22,26-imino-5,22(N)-cholestadien-3 β -ol. This was confirmed by comparison with an authentic synthetic specimen. Spectral data (U.V., IR, NMR, mass spectrometry) indicated that the carbonyl function was located on C-24. The structure of tomatillidine is therefore (25R)-22,26-imino-5,22(N)-cholestadien-24-one-3 β -ol (Y. Sato, C. Djerassi, E. Bianchi, and H. Budzikiewicz).

STEROLS FROM PARASITES. In *Taenia taeniaformis* (cat tapeworm) cholesterol exists predominantly as free cholesterol with only traces occurring as cholesterol esters. Sterols of *Trypanosoma ranarum* are being studied (Y. Sato with Theodor von Brand).

PREPARATION OF SOLASODINE GLUCOSIDE. The monoglucoside of solasodine was prepared by the interaction of solasodine with 1-bromo-2,3,4,6-tetracetyl- α -D-glucose through the Königs-Knorr synthesis and by hydrolysis of the resulting tetraacetyl derivative with barium methoxide. Chromatography over alumina afforded a fairly pure product which was submitted for testing against cancer. A number of other glucosides of steroid alkaloids are being studied (Y. Sato).

ALKALOIDS FROM *Solanum Congestiflorum* (NATRI). *Solanum congestiflorum* (natri) a plant of South American origin affords four closely related steroid alkaloids. The main component has been identified as (25R)-22,26-imino-5 α -cholest-22(N)-en-3 β -ol, the structure of which has been established unambiguously. The others present in small quantities are suspected to be the epimeric hydroxy and oxo derivatives (Yoshihiro Sato and Yoshio Sato).

SYNTHESIS OF HETEROCYCLIC STEROIDS. The synthesis of 3'-methylisoxazolino-[17,16-d]-5-androsten-3 β -ol has been realized in high yields (85-90%) by an elegant one step procedure involving oximation and concomitant ring closure of 16 α -chloro-5-pregnene-20-one-3 β -ol acetate. It consists of treating the readily available 16-chloropregnene acetate with hydroxylamine hydrochloride in pyridine at 105° for 24 hours (Yoshio Sato and H. Kaneko).

PHOTOCHEMICAL REACTIONS OF STEROIDAL ALKALOIDS. The irradiation of N-nitrosatomatidine in ethanol yields tomatidine but N-nitroso-solasodine fails to give solasodine. Instead, a new as yet unidentified substance is obtained. Irradiation of pseudotomatidine B in cyclohexane affords one product while in alcohol a number of substances are obtained (Yoshihiro Sato and Yoshio Sato).

ASSAY OF DOPAMINE- β -OXIDASE ACTIVITY IN SYMPATHETIC TISSUE. A micro method for the determination of dopamine- β -oxidase activity has been developed based on the release of tritium labeled water from the enzyme substrate during the reaction. This assay has permitted the localization of this enzyme in various tissue species and within sympathetic nerves (C. R. Creveling and J. Daly, cooperating unit: Morton Levitt (LCB)).

MICROBIOLOGICAL HYDROXYLATION OF STEROIDAL ALKALOIDS AND SAPOPENINS. An enzymatic system capable of catalyzing the hydroxylation of the sapogenin, solasodine, to form 7 β -hydroxysolasodine has been isolated from the mold, *Helicostylum piriforme* (C. R. Creveling and Y. Sato).

METABOLISM OF C¹⁴-LABELED STEROIDS BY ADRENALS FROM RATS WITH A MAMMOTROPIC PITUITARY TUMOR. The adrenal glands of rats bearing a transplantable mammotrophic pituitary tumor are enlarged up to 10 times normal size due to very high levels of ACTH. *In vitro* incubation of this adrenal tissue with progesterone-4-C¹⁴ or deoxycorticosterone acetate-2-C¹⁴ results in low yields of corticosterone and aldosterone with an accompanying increased production of two metabolites tentatively identified as 18-hydroxy derivatives of deoxycorticosterone and corticosterone. Present data suggests that the normal metabolic pathway of progesterone in this tissue is bypassed in favor of these 18-hydroxy compounds (D. F. Johnson; R. W. Bates (LNE)).

ADRENAL METABOLISM OF THE MONGOLIAN GERBIL. Chromatographic analysis of pooled blood samples indicate little or no circulating free adrenal steroids in this animal. Preliminary results of *in vitro* incubations of adrenal tissue with progesterone-4-C¹⁴ indicate a different steroid pattern compared to that of the rat. Investigations are being carried out to de-

termine any possible relationship between the steroid metabolism of the Gerbil and the low water loss observed in the animal (D. F. Johnson and cooperating unit: H. Y. C. Wong, Howard University).

PROGRAMMED GRADIENT ELUTION CHROMATOGRAPHY OF STEROIDS. A flexible and efficient method of producing concentration gradients for column chromatography of steroid mixtures using a punch-tape mechanism was described earlier. The utility of this system has been increased by addition of a Friden Auxiliary Tape Punch Console, which allows the investigator to change programs as the elution process progresses, rather than using pre-punched tapes (D. F. Johnson).

STEROL BIOSYNTHESIS IN PLANTS. Mevalonic acid-2-C¹⁴ and methionine-methyl-C¹⁴ were incubated with leaf cuttings from soybean seedlings in an aqueous medium. Radioactive transformation products were isolated by thin layer chromatography. The presence of stigmasterol and β -sitosterol has been established, and microchemical data indicates that at least two other radioactive metabolites are related stanols. The results obtained suggest a precursor relationship between these completed structure sterols *via* hydrogenase and/or dehydrogenase enzyme systems (D. F. Johnson and J. A. Waters).

ANODIC DECARBOXYLATION OF CARBOXYLIC ACIDS. Various glycidic acids are being oxidized in methanol at a platinum anode to study the mechanism of decarboxylation with reference to the opening of the epoxide group. The two major products (α,β -unsaturated ketone and methoxy ketone) suggest ring opening *via* carbonium ion intermediates from two possible pathways (J. A. Waters).

LABORATORY OF MOLECULAR BIOLOGY

Relation Between Structure and Function of Glutamate Dehydrogenase from Bovine Liver

1. Chemical structure of the subunits of GDH—The N-terminal amino acid of glutamate dehydrogenase is L-alanine. The C-terminal amino acid is L-threonine. Hydrolysis by trypsin or cleavage of the peptide chains by cyanogen bromide indicates that glutamate de-

hydrogenase is composed of a single type of sub-unit with a molecular weight of 50,000.

2. Molecular weight of enzymically active units—The polymeric form of glutamate dehydrogenase which exists above 2 mg per ml in solution has a molecular weight of 2,000,000 and is highly asymmetric. The molecular weight of the enzymically active monomeric unit catalytically active for glutamate and alanine is about 400,000 and may, under extreme circumstances, disaggregate to forms of lower molecular weight.

3. Immunological studies—A theory has been proposed to account for the separation of three immunologically different forms of the enzyme in rapid equilibrium. In addition antigenic relations between glutamate dehydrogenases from different organs of beef, different mammalian sources, and microorganisms have been studied. The "alanine form" of glutamate dehydrogenase has been found to be catalytically active for other monocarboxylic L-amino acids such as leucine, isoleucine, methionine and valine as well as the amino acid analogs, norvaline and norleucine. The "glutamate form" appears to be active only for L-glutamate and not for other dicarboxylic amino acids.

4. Allosteric effects—The allosteric effects observed with the glutamate dehydrogenase system appear to be only noncooperative. These findings have theoretical implications for the kinetic model of allosteric interactions recently developed. (G. Tomkins, E. Appella, K. L. Yielding, M. W. Bitensky, N. Talal).

Enzyme Induction in Bacteria

Kinetic studies indicate that β -galactosidase is induced prior to thiogalactoside transacetylase on enzyme in the same operon. On deinduction β -galactosidase synthesis stops before that of the acetylase. These results have been interpreted in terms of the direction of reading the messenger RNA of the lactose operon. (G. Tomkins, D. Alpers)

Enzyme Induction in Mammalian Cells

Studies of hydrocortisone induced tryptophan pyrrolase and tyrosine transaminase indicate that partial regulation of the synthesis of

these enzymes occurs at the level of messenger RNA translation rather than transcription. Additional studies indicate that the repressor operating at the translational level has a rapid rate of turnover, depends on RNA synthesis and is probably a protein. Complementary studies indicate that ribosomes in liver cytoplasm primarily exist attached to membranes in the form of polyribosomes and that a small number are unattached to the membrane. No intrinsic difference between these ribosomes was found nor was their rate of synthesis different. The turnover time of ribosomal RNA has been estimated to be 5 days. (G. Tomkins, John Loeb, B. Peterkofsky, S. Hayashi, L. Garren)

Serine Transacetylase

An enzyme has been isolated which transfers the acetyl moiety from acetyl coenzyme A to the amino group of L-serine. The enzyme has been partially purified and some of its characteristics have been studied. In addition, acetyl serine has been identified as a component of certain proteins in the cytoplasm of *E. coli*. (G. Tomkins, N. Kredich)

Mechanism of Action of Steroid Hormones

The major portion of these studies has been done on minimal deviation hepatomas carried in tissue culture. Steroid hormones were observed to induce tyrosine α -ketoglutarate transaminase in the cultured cells. The kinetics of induction, dose-response curves, media requirements, and response to inhibitors of various types were then studied. The morphology of the cells was studied by light and electron microscopy. (E. Brad Thompson)

Active Transport of Amino Acids in *Salmonella Typhimurium*

Further work has been done on the transport mechanism for amino acids. The formation of intermediates during active transport, between lipids and amino acids has been demonstrated. The nature of these intermediates is such to justify a possible role in active transport, i.e., the amino acid is unchanged and can be separated from the lipid by very mild treatment with alkali; the intermediates are formed very

rapidly in very small amounts and they are lipid soluble. There is a suggestion that each might correspond to a phospholipid with a specific conformation of fatty acids.

The whole lipid fraction and the amino acid intermediates are under further investigation in order to characterize better their structures and roles. Mutants missing various permeases will be used in the process. (Giovanna Ames)

Amino Acid Incorporation into Protein in Cell Free Systems: Influence of Ordered Structures Between Purine Ribosides and Polyribonucleotides on Messenger Activity

Monomeric nucleic acid components (nucleotides, nucleosides or purines) are capable of forming regular ordered structures with complementary polyribonucleotides (Miles, Howard and Frazier). These observations suggested that monomer-polymer interactions might be involved in some biological control mechanisms, since the secondary structure of nucleic acids is important for their function. To test this possibility we examined the effect of purine ribosides on polyribonucleotide directed amino acid incorporation in cell free systems from rat liver and *E. coli*. The ability of poly U to function as messenger RNA can be reduced or abolished by complementary ribosides (adenosine or 2-6 diamino purine). The degree of inhibition is a function of temperature and of concentration of riboside. Qualitatively good correlations are observed between conditions favoring inhibition in the biological system and those required for the formation of helices as measured by physical chemical methods. The site of action of the ribosides was delineated by examining their influence on the binding of poly U to ribosomes and by studying their effect on the rate of degradation of poly U in the systems. The ribosides exert dual effects upon poly U. They prolong its lifetime in the system and, at the same time, by decreasing its binding to ribosomes they suppress its activity as messenger RNA. Preliminary experiments with a copolymer of UC indicate that ribosides of complementary bases can influence amino incorporation directed by copolymers. When amino acid incorporation is directed by endogenous natural messenger RNA,

small temperature dependent inhibitions by purine ribosides are observed. (E. S. Maxwell, H. T. Miles, F. B. Howard)

Helix Formation Between Polynucleotides and Purine Monomers

Purines, purine nucleosides and purine nucleotides of appropriate structure interact with polynucleotides to form two or three-stranded helices analogous to those formed between pairs of polynucleotides. Base pairing specificity is observed in these interactions. Pyrimidines and their derivatives appear to be unable to serve as monomer components in such interactions. The phenomenon has provided a convenient model system for the study of factors affecting nucleic acid stability and may be of importance in the control of some biologically important reactions. The method of infrared spectroscopy in aqueous solutions was further developed to study the formation and dissociation of these complexes. (H. T. Miles, F. B. Howard; biochemical aspects of the problem were studied in collaboration with E. Maxwell and M. F. Singer)

Chemistry of Isoguanosine

Isoguanosine in aqueous solution forms a regular ordered structure which is highly asymmetric and presumably helical. The phenomenon was studied by infrared and ultraviolet spectroscopy, optical rotation, and concentration dependence of viscosity. The tautomeric structure and site of protonation of the nucleoside have been studied by infrared and nuclear magnetic resonance spectroscopy.

The 5'-phosphate of isoguanosine has been synthesized by the cyanoethylphosphate method, and preliminary experiments indicate that it does not undergo self-structure formation. (R. V. Ravindranathan)

Optical Properties of Nucleic Acid

We have shown that for a random sequence of bases, it will be true quite generally that the hyperchromism of DNA at a given wavelength will be a quadratic function of the base composition of the region undergoing denaturation. Thus, three parameters at each wavelength

suffice to predict the contribution to hyperchromism of a region of known base composition. We have obtained the parameters at each wavelength by measurement of a large number of bacterial DNA hyperchromic spectra. It is possible to use the equations to determine the composition of each region of a denaturing DNA molecule as it denatures, and thus to obtain a "map" of the DNA. Important evidence concerning the structure of viral DNA's and particularly of λ DNA, has been obtained by this method.

We have also shown that the helix and coil spectra of DNA can be analyzed separately in a manner similar to that used for hyperchromism. The method presently is sufficiently precise to permit accurate determination of the base composition and concentration of an unknown DNA from its native or high-temperature spectra alone. If both spectra are known an accurate determination of the amount of denatured material present in the original sample can also be made. (Gary Felsenfeld, Shalom Z. Hirschman)

Polyadenylic Acid at Ph 7

It has already been shown in this laboratory that the neutral poly A structure undergoes thermal disruption by a completely non-cooperative process, so that the process can be analyzed well by means of the van't Hoff method, which yields an "apparent ΔH " for the reaction. We have accurately redetermined the value of ΔH for poly A, and compared it with that for the oligonucleotide series. Aside from what appear to be polyelectrolyte swelling contributions, values of ΔH for all members of the series are the same. It is possible to conclude that poly A is composed of small independently denaturing units, about two nucleotides long each, and that there is no long range ordered, single stranded structure. (Marc Leng, Gary Felsenfeld)

Internal Proteins of Bacteriophage T2

Other workers have demonstrated that at least two proteins are released from bacteriophage T2 by osmotic shock (e.g., Hershey, A. D., Virology 1: 108 (1955); ibid., 4: 237

(1957)). The proteins are distinguishable by their solubility in cold trichloroacetic acid.

Current work has separated these components on acrylamide P-60 columns. The acid-soluble protein has also been chromatographed as a single peak on DEAE-cellulose. The acid-insoluble protein has resisted further purification procedures, largely due to irreversible surface adsorption on most materials.

Pulse-labelling of T2-infected *E. coli* indicates both proteins are made approximately coordinately during infection, their synthesis beginning within three minutes after infection starts. (Steven B. Zimmerman, Gary Felsenfeld)

Ribonuclease Activity of Pancreatic Deoxyribonuclease

It was previously found that a small but significant RNase activity persisted in pancreatic DNase through various chromatographic procedures. Further purification attempts failed to separate the two activities. However, it was possible to inactivate differentially the RNase or the DNase. N-Bromosuccinimide completely destroyed DNase activity without altering RNase levels; sodium iodoacetate reduced RNase activity to levels indetectable by the ultrasensitive assay while recovery of DNase was 70-90%. (Steven Zimmerman)

Viscosity Studies of DNA

High precision viscosimetric study of bacteriophage DNA.

The intrinsic viscosity of DNA (salmon sperm, calf thymus T4 bacteriophage) in the presence of uni-univalent electrolytes decreases in a continuous manner as the ionic strength is increased and then attains a limiting value at about 0.5 M.

The concentration dependence of the reduced viscosity may be abolished and regenerated by the introduction and removal of trace amounts of basic proteins, polypeptides and polyamines.

In contrast to earlier reports the marked sensitivity of the intrinsic viscosity of the DNA molecule to its ionic environment represents typical polyelectrolyte behavior. The viscosity behavior observed with the deliberately

contaminated DNA samples resembles most of that reported in earlier viscosity studies, indicating that a large body of previous work on DNA probably was performed on impure material. (Philip D. Ross)

Three-Stranded Poly (A+2U) Complex

Calorimetric measurements of the heat of the addition of the second strand of poly U to poly (A+U) to form the three-stranded poly (A+2U) complex in 0.1 M and 0.5 M NaCl at 24° and 37° are reported. A value of $\Delta H = -3800$ cal. (mole of poly (A+2U) formed)⁻¹ was found to be fairly insensitive to the experimental conditions employed. The heat of the addition of the third strand to the preformed poly (A+U) helix is considerably less exothermic than the heat of reaction between poly A and poly U to form poly (A + U). The insensitivity of the heat of addition of the third strand (poly U) to changes in salt concentration and temperature lends qualitative support to the earlier hypothesis that the major portion of the variation of the ΔH of the poly A and poly U reaction with salt concentration and temperature arises from differences in the conformation of poly A.

Combining the information obtained in this study for the ΔH of the reaction forming poly (A+2U) with previous data for the ΔH of the formation of poly (A+U) indicates that the conversion of poly (A+U) to poly (A+2U) is opposed by an enthalpy change which increases with temperature. Extrapolation of these values to 52° where poly (A+U) is transformed to poly (A+2U), in 0.5 M NaCl leads to a value of $\Delta H = +3800$ cal. (mole (A+2U) formed)⁻¹ and $\Delta S = 11.5$ cal. (mole of (A+2U) formed)⁻¹ deg.⁻¹. The latter value of 11.5 entropy units derived from the experimental data may provide a good estimate for the entropy change of a single random coiled polynucleotide entering a helical structure. Similar values have been previously found for poly (A+U) and DNA. It is concluded from the calorimetric data that the driving force for the interesting poly (A+U) to poly (A+2U) conversion reaction must be the favorable entropy change. (Philip D. Ross)

Protein Structure

A correlation has been demonstrated between the alpha helix content of globular proteins as determined by X-ray diffraction and optical rotatory dispersion measurements and their amino acid composition. Specifically, there appears to be a linear relationship between the helix content of a variety of proteins and their percent composition of the amino acids, serine, threonine, isoleucine, valine, cysteine and proline. (David R. Davies)

Structural Studies on Nucleic Acids, Polynucleotides and Nucleotides

The interaction of dyes of the acridine class, such as proflavine and acridine orange with DNA have been investigated. It has been shown that under conditions of high hydration, diffraction patterns can be obtained from fibers of dye-DNA complexes which show marked changes from the diffraction patterns of sodium-DNA fibers. These changes consist of an increase in the layer line spacing and a disorder of the diffraction pattern.

A study of the optical transforms of various simple models has shown that there are two which are consistent with this type of change in the diffraction pattern. In one of these the dye is intercalated between the base pairs of the DNA, and in the other the dye binds on the outside of the DNA and produces large changes in the helical screw of the DNA. Taken in conjunction with measurements on the increases in length of the fibers as a function of the layer line spacing and with the observations of other workers, these results have been interpreted as providing support for the intercalation hypothesis. (David R. Davies and David Neville)

Stable Heterogenotes of Bacteriophage T4

T4 phages carrying two copies of the rII region have been described (see 1964 Annual Report). Our recent work has been directed at understanding the nature of this genetic anomaly. A series of three-factor crosses, with genetic markers straddling the rII region, has shown that the two rII genes are closely linked. The structure may thus be similar to that of genetic duplications in higher organisms, and

is definitely unlike that of normal (unstable) T4 heterozygotes.

Recent genetic evidence (Weil *et al.*) suggesting that the duplication may extend well beyond the rII region is in course of investigation by physical chemical methods in order to see whether there is a correlation between the size of the DNA of the mutant phage and the extent of the duplication. (Martin Gellert, Philip D. Ross)

Biochemical Control Mechanisms in Histidine Biosynthesis

Further work has been done on the intermediates, enzymes, genes and control mechanisms of histidine biosynthesis.

1. A new intermediate in the pathway of histidine biosynthesis has been isolated, and shown to have the structure phosphoribosyl-AMP. Two enzymes have been described: PR-ATP-pyrophosphohydrolase, which forms PR-AMP from PR-ATP, the first intermediate of the pathway, and PR-AMP, 1,6-cyclohydrolase, which opens the purine ring of PR-AMP, converting it to the next intermediate of the pathway. (D. W. E. Smith, B. N. Ames)

2. Two enzymes of histidine biosynthesis have been purified from *Salmonella*: the first enzyme of the pathway and IAP-transaminase. The transaminase is a single chain of 67,000 MW. with a C-terminal valine and an N-terminal serine. The pure enzymes are necessary in the study of the biochemical basis of polarity, modulation, feedback inhibition, and repression. (R. G. Martin, M. J. Voll, E. Appella, B. Ames)

3. An extensive study of polarity mutants in the transaminase gene indicates that most mutants are polarity mutants. The theory of modulation and polarity has been developed further. (R. G. Martin)

4. The important pool in repression of the histidine biosynthetic enzyme appears to be that of histidine-sRNA rather than that of histidine. This conclusion is based on the isolation of various mutants with decreased histidine activating enzyme that accumulate histidine yet are derepressed for the histidine biosynthetic enzymes. (John Roth and P. E. Hartman of John Hopkins, and B. Ames)

5. One aspect of the mode of action of the widely used herbicide amitrole (aminotriazole) has been determined. The compound inhibits imidazole glycerol phosphate dehydrase, one of the enzymes of histidine biosynthesis. (J. L. Hilton, P. C. Kearney of U.S.D.A., and B. Ames)

Chemistry and Genetics of Hemoglobin and Other Proteins

In order to study the forces that hold the sub-units of hemoglobin together, lysyl residues were converted to homoarginyl residues by conversion of ϵ -amino groups to ϵ -guanidino groups. Guanidinated human adult CO-hemoglobin has the absorption spectrum of untreated hemoglobin and forms large prismatic crystals. Solutions of this derivative are considerably more resistant than unmodified hemoglobin both to denaturation and to dissociation of high pH. The structure of the addition compound of benzil and arginine has been established. This chemical modification results in resistance of the arginyl site to tryptic hydrolysis. (H. A. Itano, A. J. Gottlieb, K. Toi)

Mechanism of Lambda Bacteriophage Induction

We are encouraged to believe, from a study of a process called "curing", that the viral functions involved in lysogenic induction may be similar or identical for bacteriophages differently repressed. In curing, a superinfecting phage appears to provide the necessary functions for excising prophage from some bacterial nuclei without irrevocably damaging either these nuclei or the cells in which they are located. Excision of prophage by a lytic superinfecting phage without concomitant expression of prophage functions can also be demonstrated.

A search among mutants of the well-studied bacteriophage lambda, has revealed one which appears incapable of curing yet which retains its other functions. Once we are in a position to specify genetic blocks in functions unique to the induction process we are in a position to understand this remarkably precise rearrangement of genetic material. (M. Yarmolinsky)

Host-Induced Modification

An investigation has been made of the growth restriction of *E. coli* strains against the

host modified phage T*4 and also the permissiveness of *Shigella dysenteriae* strains (Sh) for the phage.

On the basis of previous results, it has been postulated that an RNA-inhibited endonuclease is responsible for restricting ability of *E. coli* B against T*4, containing nonglucosylated DNA. This hypothesis is now under test (T. Fukasawa)

CLINICAL INVESTIGATIONS

INTRODUCTION

The clinical research program of NIAMD received a severe setback at the outset of this fiscal year in the loss of Dr. Joseph J. Bunim, who had so ably supervised clinical investigations since before the Clinical Center was opened. His post as Chief of the Arthritis and Rheumatism Branch remains unfilled at this writing, but it is anticipated that the name of a successor will soon be formally announced.

In September 1964, under the auspices of the American College of Physicians, the Arthritis and Rheumatism Branch, NIAMD, conducted a five-day course at the Clinical Center entitled "Current Clinical and Laboratory Investigations in the Rheumatic Diseases." Planning for this teaching exercise had been initiated by Dr. Bunim, and the project was carried through by members of the staff. An outstanding group of 19 guests and 11 NIH scientists served on the faculty. Fifty physicians representing all areas of the country were registered and participated in the course.

In November 1964, NIAMD and the Clinical Center served as hosts for the American Rheumatism Association interim scientific meeting. This tradition had been established some years ago by Dr. Bunim, and was carried on this year by other members of the staff.

During the period that the Arthritis and Rheumatism Branch has been without a chief, a reduction in its clinical activity has been unavoidable. This fact, and the temporary absence of Dr. J. E. Seegmiller, have resulted in a temporary decrease in the Institute's patient-care activities. During the period March 1, 1964 to February 28, 1965, NIAMD admitted 508 patients for a total of 18,593 hospital days. The

average patient stay was 37 days, and the average census 70% of capacity. During the same period, there were 2318 outpatient visits for study and/or treatment.

ARTHRITIS AND RHEUMATISM BRANCH

Clinical Observations on Rheumatic Diseases

ASSOCIATION OF LYMPHOMA AND SJÖGREN'S SYNDROME. Through the years a number of patients with Sjögren's syndrome have been admitted to the Clinical Center for observation. It was noted that an unexpectedly high percentage of these patients manifested lymphadenopathy with malignant degeneration. Four such cases were reported in 1964. Two of these patients are still alive, and are under continuing study at the Clinical Center. During the past new cases were seen, in one of whom a biopsy showed benign lymphatic hypertrophy. The other was diagnosed as lymphoma. In addition, pathologic material on two similar cases has been obtained from other institutions for comparative study. This descriptive study is of particular interest because of the possible involvement of auto-immune mechanisms in the etiology of lymphoma on the one hand, and of Sjögren's syndrome on the other. (Dr. Talal)

CLINICAL OBSERVATIONS ON JUVENILE RHEUMATOID ARTHRITIS. In the decade since the Clinical Center opened, 60 patients with juvenile rheumatoid arthritis have been admitted. These medical records have all been reviewed, and as many of the patients as possible have been reexamined. The data on the natural history of juvenile rheumatoid arthritis, as exemplified by this experience, are being collected and analyzed in the light of a review of the existing literature on the subject. It is hoped that this study will provide useful background material and guidance for future clinical investigation of the syndrome. (Drs. Washburn and Alepa)

CHRONIC EFFECTS OF ALLOPURINOL ON GOUT. Allopurinol is effective as an inhibitor of xanthine oxidase, and is now available for experimental use in the treatment of gout. Its effect is to decrease the conversion of xanthine and hypoxanthine to uric acid, so that blood

urate levels fall and xanthine and hypoxanthine levels rise. It offers promise as a drug for the management of gout if it can be well tolerated over long periods. In the present study, four patients with chronic gout, three of whom had measurable tophi, were started on 400 milligrams of allopurinol per day. So far, the drug has been well tolerated for periods up to a year, with an effective decrease in plasma urate concentrations. Xanthine excretion in the urine increased, but with the maintenance of an adequate fluid intake, there was no evidence of renal stone formation. The patients are still on treatment and under observation; if the promising results of the study to date continue to apply in the future, it would appear that this drug will have therapeutic potential in those patients who for one reason or another cannot be properly managed with uricosuric agents. (Drs. Alepa and Seegmiller.)

Studies in Serology and Immunochemistry

ANTIBODIES TO HUMAN GAMMA GLOBULIN AMONG PATIENTS WITH RUBELLA. Thanks to the cooperation of the Perinatal Research Branch, NINDB, paired serum samples were obtained on each of 53 patients infected during a recent rubella outbreak on an Arctic island. These sera have been examined with the bentonite flocculation test in order to evaluate further the significance of reports indicating the presence of rheumatoid factor in patients with rubella complicated by arthritis. While low titers of rheumatoid factor were found in 7 of the 53 initial serum samples, higher titers were present in 39 of the 53 specimens taken five months later. This represented an increase in titer of 3 tubes or more in 69% of the samples. These results indicate that an acute self-limited viral infection, like chronic infections in man, can give rise to positive bentonite flocculation tests. (Drs. Alepa and Monif (NINDB).)

GENETICALLY DETERMINED GAMMA GLOBULIN POLYMORPHISM IN CHIMPANZEES. The genetically determined antigens (Gm and Inv) found in human gamma globulin have been useful markers in the study of immune mechanisms. These antigens can be recognized only by the use of specific antisera obtained from rare pa-

tients who develop sensitization to these factors. No animal sera with anti-Inv(b) specificity have so far been prepared. Therefore, a study has been undertaken to determine the distribution of Gm and Inv types among chimpanzees, in hopes that by immunizing one animal with gamma globulin from another, antisera can be produced at will which will be of use in human genetic studies. (Dr. Alepa with Dr. Terry (NCI) and Dr. Moor-Jankowski (Emory Univ.).)

INTERACTIONS BETWEEN POLYPEPTIDE CHAINS OF GAMMA GLOBULIN. Previous studies in this and other laboratories have demonstrated that IgG antibody molecules can be separated *in vitro* into heavy and light polypeptide chains. These chains can then be induced to recombine yielding an immunologically active antibody molecule. It has thus been possible to investigate various factors, biological and chemical, which govern the makeup of naturally produced antibody molecules. In the present study fragments derived from the dissociation of nonspecific rabbit IgG protein was allowed to recombine with fragments derived from a specific antibody directed against the 2,4-dinitrophenyl hapten. The polypeptide chains were separately identified by suitable labeling with I¹³¹ and I¹²⁵. It was found that in the absence of the DNP hapten, chance alone governed the recombination of heavy and light chains. In the presence of the hapten group, however, recombination of heavy and light chains from the specific antibody molecule was favored. These findings indicate that both the heavy and light chains probably participate in the formation of the specific immunologically active site, and may throw further light on the mechanism of induction of antibody synthesis *in vivo*. (Drs. Metzger and Mannik.)

In a related study, a homogenous abnormal immunoglobulin was obtained by purification of human myeloma protein. Heavy and light chains from this protein were purified, and then allowed to recombine in the presence of polypeptide chains derived from other antibody molecules. By the use of I¹³¹ and I¹²⁵ labels to trace specific polypeptide chains, it was found that heavy chains tended to recombine with light chains derived from the same origi-

nal antibody molecule. (Dr. Mannik with Dr. H. Gray (Rockefeller Inst.).)

CHARACTERIZATION OF A HUMAN MACROGLOBULIN. A macroglobulin (IgM) obtained from a patient was highly purified by gel filtration. The molecular weight of this protein was found to be 892,000, and subunits formed by partial dissociation had a molecular weight of 185,000. This is consistent with the view that 5 subunits are polymerized to form one macroglobulin molecule. Subunits were dissociable into heavy and light chains, as are other immunoglobulins, with a yield of two heavy and two light chains per subunit. The heavy chains derived from IgM were of greater molecular weight than those derived from IgG; only part of the difference can be accounted for on the basis of carbohydrates. (Drs. Metzger and Miller.)

METABOLISM OF ALTERED IMMUNOGLOBULINS. Purified rabbit gamma globulin, after labeling with I^{125} , was readministered to rabbits and its metabolism followed by standard methods. This experiment was done with unaltered gamma globulin and with the protein after reduction and alkylation. The destruction of the disulphide bridges between polypeptide chains of these immunoglobulins did not cause the molecule to break up into fragments *in vivo*. Surprisingly enough, the altered molecule, which might have been expected to be less stable, had a longer half-life in the recipient animal's plasma than did the native antibody. (Drs. Cohen and Mannik.)

Biochemical Studies

PROTEIN SYNTHESIS IN THE SPLEEN. Subcellular fractions prepared by differential centrifugation of homogenates of rat splenic tissue are being studied with regard to their activity in protein synthesis. It is hoped that with further development, these methods will yield a cell-free preparation suitable for the investigation of antibody synthesis *in vitro*. A polyribosome preparation obtained from splenic tissue has been found to incorporate radioactivity from labeled amino acids. The reaction depends on magnesium and another soluble co-factor, and is inhibited by puromycin or by ribonuclease. The nature of the newly-formed protein

is not yet known, but experiments to study its relationship to gamma globulin are in progress. (Dr. Talal.)

IMMUNOCHEMICAL STUDY OF GLUTAMIC DEHYDROGENASE. Preparations of glutamic dehydrogenase from different sources have been tested immunochemically and electrophoretically for structural differences. The sources of glutamic dehydrogenase were six different bovine tissues, and one tissue, liver, from eight different vertebrate species. All preparations of beef glutamic dehydrogenase appeared to be identical. The dehydrogenase preparations from the livers of various species could be distinguished, although in some cases no immunochemical difference could be shown. A preparation of glutamic dehydrogenase from a bacterial source, however, did not cross-react at all with these preparations. This study is of interest in relating biochemical differences to species which have developed during the course of evolution. (Drs. Talal and Tomkins (A-LMB).)

STUDY OF HUMAN LACTATE DEHYDROGENASE ISOZYME PATTERN. Preparations of lactate dehydrogenase prepared from the red blood cells of 1,200 hospital patients in the Washington, D.C. area have been studied by starch gel electrophoresis. The cell donors included 600 Negroes and 600 Whites. The electrophoretic mobilities of lactate dehydrogenase preparations demonstrate a genetically controlled polymorphism which can be analyzed in terms of two genetic loci. Electrophoretic patterns show up to five lactate dehydrogenase fractions, the relative quantities of which vary from one individual to another; these patterns can be explained on the basis of the formation of tetramers from monomeric units not all of which are identical. Dissociation of the subunits of lactate dehydrogenase can be accomplished *in vitro*, and recombinants prepared artificially. (Dr. Vesell.)

BIOCHEMISTRY OF ACTH. The effect of ACTH on the adrenal gland of the intact rat is being investigated. Steroid secretion is measured by the analysis of adrenal vein blood. Radioisotope incorporation into the glandular tissue serves to measure the rates of protein and nucleic acid synthesis. Results to date show that agents which prevent protein synthesis,

such as cycloheximide and puromycin, prevent the effect of ACTH in stimulating steroid secretion. Inhibition of nucleic acid synthesis by actinomycin D does not have this effect. Continuation of these investigations promises to throw further light on the intimate mechanisms by which polypeptide hormones can control the cellular metabolism in the target organ. (Dr. Garren.)

HORMONAL REGULATION OF ENZYME INDUCTION. Hydrocortisone administered to rats causes an increase in the rate of synthesis of the liver enzymes, tryptophan pyrrolase and tyrosine transaminase. Since the stimulation of these enzymes occurs within 30 minutes after hormone administration, they have been studied as a model of hormone regulation of protein synthesis.

Studies utilizing this system have indicated: 1) that DNA (mRNA) dependent RNA synthesis is involved, since enzyme induction is prevented by actinomycin D which blocks RNA synthesis; 2) that the template RNA for these enzymes is stable, since non-induced enzyme synthesis continues for at least six hours in the presence of complete inhibition of further RNA synthesis. (If mRNA turned over rapidly, as in bacteria, enzyme synthesis would cease within minutes); 3) that following enzyme induction another regulatory mechanism is activated which inhibits further synthesis of these enzymes. This inhibition apparently does not involve the level of mRNA, which is apparently stable, but appears to operate directly at the level of the cellular protein synthetic machinery (translation of mRNA). The abrupt decrease in the rate of enzyme synthesis which usually occurs four hours after hormonal stimulation can be prevented by administration of actinomycin D. This suggests that a DNA dependent RNA is made at this time which inhibited further enzyme synthesis. The possibilities of this model system for investigating mechanisms of regulation of protein synthesis have not been exhausted. (Dr. Garren.)

BIOCHEMISTRY OF CARBON-FLUORINE BOND. A soil bacterium which can utilize fluoroacetate as its sole source of carbon has been isolated. This organism possesses a defluorinating enzyme which has been partially purified, and the mechanism of the reaction that it cata-

lyzes is being studied. The fluorine is replaced by a hydroxyl group, with the liberation of fluoride ion. Fluoroacetate and iodoacetate are similarly attacked, although at a slower rate. The enzyme, however, does not attack the carbon-fluorine bond in other compounds. Because of the increasing use of fluorinated analogues of hormones and metabolites as therapeutic agents, further investigation of the biochemistry of fluorine-carbon compounds may be of pharmacologic interest. (Dr. Goldman.)

BIOCHEMISTRY OF MAPLE SYRUP URINE DISEASE. The biochemical properties of the decarboxylase for the keto-acids derived from leucine, isoleucine, and valine are being investigated in tissues obtained from animal and human sources, including fibroblasts cultured *in vitro* from skin explants of a patient with Maple Syrup Urine Disease. (Dr. Seegmiller with Drs. Westall and Dent, Univ. College Hospital, London.)

BIOCHEMISTRY OF CYSTINOSIS. The biochemical abnormalities leading to the deposition of cystine crystals in the tissues of patients with cystinosis are being investigated by studies of the metabolism of the sulfur-containing amino acids both *in vivo* and *in vitro*. Comparisons will be made of the lethal childhood variety of this disease with the adult form of the disease, which has been found in three siblings, and appears to be a benign disorder. (Dr. Seegmiller with Dr. P. Leitman (Johns Hopkins), Dr. P. Frazier (NDI) and Dr. D. Shotton (Medical Center, Lynchburg, Va.)

ROLE OF CRYSTALS OF MONOSODIUM URATE IN THE GENESIS OF GOUTY ARTHRITIS AND A MECHANISM FOR THE THERAPEUTIC ACTION OF COLCHICINE. The mechanism by which crystals of monosodium urate give rise to the acute attack of gouty arthritis is being investigated. The possible role of kinin peptides in this process is being explored. (Dr. Seegmiller with Dr. Klinenberg (Johns Hopkins), and Drs. Melmon, Webster and Sjoerdsma (NHI).)

GASTROENTEROLOGY UNIT

Small Intestine

WHIPPLE'S INTESTINAL LIPODYSTROPHY. This study of the natural history of Whipple's disease is still in progress. One of the

patients had been treated with antibiotics and steroids for only 3-4 months before her referral to N.I.H. in 1962, but appeared to be in remission as a result. She was checked at 6-10 month intervals and did well until a relapse occurred in 1965. Studies of the morphology of her intestinal mucosa by light and electron microscopy; tests of intestinal absorptive function; and measurements of gastrointestinal protein loss are now in progress.

The other four patients in this study were treated for periods of from 2 to 3 years, and all appear to be doing well. One of these four was treated only with broad spectrum antibiotics, the others received the antibiotics plus steroids. (Drs. Leonard Lester, Robert Ockner, James Finkelstein, NIAMD; Thomas Waldmann, William G. Banfield, NCI; Paul Wertlake, CC.)

OTHER DISEASES. Studies of intestinal function and structure and studies of protein metabolism have been performed in patients with various diseases affecting the small bowel. The diseases include: scleroderma (2 patients); amyloidosis (7 patients); inflammatory disease due to ileitis and ileocolitis (2 patients); gluten-sensitive enteropathy (10 patients); lactase deficiency (5 patients); Hodgkin's disease (1 patient); hypogammaglobulinemia (7 patients); glycolipidosis of Fabry (2 patients); and vascular disease of the small intestine (2 patients).

Excessive loss of protein into the gut may occur in almost any one of these diseases and when therapy is available for the underlying disease it appears capable of rapidly reversing abnormal protein exudation. The intestinal bacterial flora exert a profound influence on intestinal function and several instances of reversal of fat malabsorption by alterations of bacterial flora are available among these studies. An unusual example of apparent intestinal lactase deficiency in identical twins, 63 years of age, was discovered during this work. (Drs. Leonard Lester, Robert Ockner, James Finkelstein, NIAMD; Thomas Waldmann, NCI; Paul Wertlake, CC.)

PATHOLOGIC PHYSIOLOGY OF THE INTESTINAL MUCOSA. Incubation of hamster small intestine in Krebs-Ringer-phosphate buffer containing 40 mM L-tryptophan for 20

minutes produced a lesion in the columnar absorptive cells characterized by apical displacement of the nuclei; crescentic shape to nuclei; extreme vacuolization of the basal half of the cell; multiple projections of cytoplasm from the basal half of the cell; and separation of the cell from, with possible disruption of, the basement membrane. Control studies in which the L-tryptophan was replaced by its D-form, glycine, or L-forms of eleven other amino acids revealed no changes similar to those produced by L-tryptophan. The severity of the lesion was proportionally related to time of exposure to the L-tryptophan and to the concentration of that amino acid. Biopsy specimens of human jejunal mucosa exposed to 40 mM L-tryptophan for 20 minutes developed early but typical changes of the lesion. Hamster esophagus, stomach, large intestine, pancreas, liver and kidney did not develop the typical lesion.

Intestinal transport is disrupted by the exposure to the L-tryptophan. Whether these functional and morphological effects of L-tryptophan, a natural dietary constituent, have clinical implications is under study. (Drs. Leonard Lester, NIAMD; Paul Wertlake, CC).

BIOCHEMISTRY OF THE SMALL-INTESTINE MUCOSA. The *in vitro* incorporation of acetate-2-C¹⁴ and mevalonate-2-C¹⁴ into 27-carbon sterols by scrapings of guinea pig small-intestine mucosa has been investigated. About 98 percent of the isotope incorporated into 27-sterols was detected in lathosterol plus 7-dehydrocholesterol plus cholesterol. The activity of mucosa from the distal two-thirds of the small intestine consistently exceeded that of the proximal small intestine.

Preliminary studies have shown that a commercial preparation of bile salts (sodium taurocholate, 72%, sodium glycocholate, 28%) at 4×10^{-3} M results in a profound inhibition of sterol biosynthesis from either substrate. The inhibition is greater with mucosa from the distal small intestine, the area shown by others to be most active in the transport of bile salts. (Drs. Robert Ockner and Leonard Lester, NIAMD.)

INBORN ERRORS OF METABOLISM—HOMOCYSTINURIA. The excretion of abnormal amounts of homocystine in the urine occurs together

with a clinical syndrome characterized by mental retardation, dislocated ocular lenses, cardiovascular disease, fatty liver, and other abnormalities. A patient with this syndrome was found to lack the enzyme cystathionine synthase in her liver and deficiency of this enzymatic activity appears to be the fundamental defect in the disease. (Drs. Harvey Mudd, NIMH; James Finkelstein, Filadelfo Irreverre and Leonard Lester, NIAMD.)

Additional studies have shown that this patient's apparently normal parents have approximately 35 percent of the mean control value for hepatic cystathionine synthase activity. It has been proposed that the parents are heterozygous for the defect. The patient's cousin is mentally normal but excretes homocystine in the urine. Her hepatic cystathionine synthase activity was shown to be 12 percent of the mean control value. Although her genetic status is not clear, she demonstrates that cystathionine synthase deficiency severe enough to cause homocystinuria does not necessarily cause mental retardation. (Drs. James Finkelstein, NIAMD; Harvey Mudd, NIMH; Filadelfo Irreverre and Leonard Lester, NIAMD.)

Subsequent studies have revealed that the patients with markedly subnormal hepatic cystathionine synthase activity have an impaired ability to convert the sulfur of L-methionine to inorganic sulfate. This finding indicates that the defect which was discovered in the liver represents a generalized disturbance of the body's methionine metabolism; and that cystathionine synthesis is an integral step in the major pathway for methionine catabolism in man. This study also revealed that during administration of L-methionine, the patient with cystathionine synthase deficiency excreted abnormal amounts of sulfur-containing compounds other than homocystine. The amounts of these urinary metabolites exceed that of homocystine. Their identification is in progress. (Drs. Leonard Lester, NIAMD; Harvey Mudd, NIMH; James Finkelstein, and Filadelfo Irreverre, NIAMD.)

CLINICAL ENDOCRINOLOGY BRANCH

The varied activities of the Branch have continued along similar lines as in past years, with

the chief exception of new investigations employing immunoassay of protein hormones. One member of the Branch has been assigned during this year to the Weizmann Institute in Israel, and one returned early in the year from the National Institute for Medical Research in England. The Branch has again had the benefit of being host to several visiting workers from abroad: from Belgium, England, Italy (2), Scotland, South Africa, and Sweden.

Thyroid Biochemistry

IODIDE TRANSPORT. Although many substances interfere with thyroidal iodide transport, only TSH has been known to enhance this transport. It has now been found that administration of cysteamine or cystamine to rats rapidly produces a 2-3 fold increase in iodide accumulation when organification is blocked. This effect is not mediated by the kidney or the pituitary, nor by a basic change in the iodide concentrating mechanism, since the K_m is unaffected. The effect may be produced through the $\text{Na}^+ + \text{K}^+$ -requiring, ouabain-sensitive ATPase activity, since this system can be stimulated by cystamine. A secondary anti-organification effect of these compounds ($\sim 1/50$ the activity of methylmercaptoimidazole) appears to be unrelated to the effect on iodide transport (Drs. Wolff and Rall.)

Isolated thyroid epithelial cells, produced by collagenase treatment, have been found to accumulate and organify iodide, and to possess an active ouabain-sensitive ATPase. Attempts to isolate the cell membranes in order to localize both the iodide-carrying lipid and the ATPase in the cell have thus far been unsuccessful. (Drs. Wolff and Alexander.)

In *in vivo* studies, the goitrogenic potency of various antithyroid anions has been predictable within an order of magnitude by their K_i for iodide transport measured *in vitro*. However, goiters produced by the anions are smaller than those produced by propylthiouracil in doses equally potent in blocking organic iodine formation. Furthermore, the size of the goiter produced by propylthiouracil is smaller when the anions are administered concomitantly. The mechanism of this "antigoitrogenic" property

of anti-thyroid anions is under investigation. (Drs. Wolff and Alexander.)

IODINATION REACTIONS. In further studies on the chemistry of iodination of tyrosine derivatives, neutral salt had no effect on the reaction rate. Since this should affect a reaction between two charged species, and since evidence is strong that the phenoxide is one of the reactants, it is likely that the iodinating species is I₂. Blocking the amino and carboxyl groups decreases the reaction rate. Compared to tyrosine, iodination of the methyl ester and the N-acetyl derivative are about 25% slower at pH 9.6 in carbonate buffer. An ionizable substituent, however, (e.g., glycyl-L-tyrosine) may lead to a faster rate.

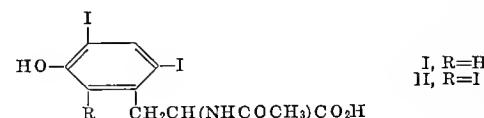
Activation energies for tyrosine and most of its amino and carboxyl derivatives were about 17 kcal mole⁻¹, but that for 3-iodo-L-tyrosine was 21 kcal mole⁻¹. The entropies of activation showed similar differences (15 versus 22 e.u.). The relation of these values to the more rapid iodination of tyrosine previously observed is under consideration. (Drs. Rall, Mayberry, and Berman—OMR.)

DEIODINATION REACTIONS. In several biological systems, deiodination of thyroxine in the presence of flavins is enhanced by ferrous ion, amino acids, and proteins. A study of this reaction from a purely chemical standpoint has shown that thyroxine is deiodinated in the presence of FMN, light, oxygen and phosphate buffer, pH 7. The reaction is first order with respect to thyroxine and is proportional to light intensity, but FMN acts catalytically. Triiodothyronine is deiodinated similarly but at a slower rate. Amino acids, amines and proteins stimulated the reaction, but the use of a metal-free system prevented the stimulation by aliphatic amino acids. Since addition of bivalent metal ions restored activity, it seems likely that chelated metal ion acts as an artificial peroxidase. Further studies with a system containing hydrogen peroxide instead of FMN confirmed this idea, since deiodination was increased by addition of ferrous ion and versene together. Independently, these agents were without effect. (Drs. Rall and Reinwein.)

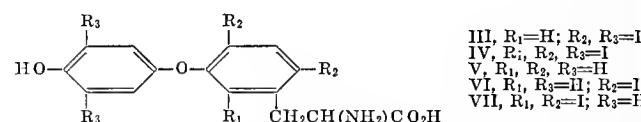
A study of deiodination of thyroxine by thyroid cell particles has been initiated. In the presence of β -mercaptoethanol at pH 7.5–8.1, deio-

dination is virtually complete. The products are iodide and three unidentified compounds, presumably iodothyronines. (Drs. Pastan and Almqvist.)

THYROXINE SYNTHESIS. In a continuation of model organic syntheses related to thyroxine formation, a number of new thyroxine analogs have been produced. Reaction of the iodinated metatyrosines I and II



with 3,5-diiodophenylpyruvic acid (DIHPPA), followed by deacetylation, gave tetra- and penta-iodinated analogs III and IV.



The poor yield from this synthesis was improved by a different approach involving etherification of the ethyl esters of I and II with di-p-methoxy-phenyliodonium bromide. Analogs V, VI and VII were also synthesized. The ability of these analogs (except V) to produce mitochondrial swelling was tested by Prof. R. Michel (College de France), who found that analogs III and IV had a behavior very like that of thyroxine. (Drs. Cahnmann, Shiba, and Höfer.)

Further studies on the reaction of DIHPPA with monoiodotyrosine (MIT) and diiodotyrosine (DIT) residues of thyroglobulin (TG) were carried out. The products differ from those of natural iodothyronine biosynthesis in that thyroxine and 3,3',5'-triiodothyronine (T_{3'}) are formed in approximately equal amounts in the model reaction, whereas thyroxine is preferentially formed *in vivo*. Labeled thyroxine and T_{3'} could be detected only if slightly iodinated TG-¹²⁵I was used. This suggests that DIHPPA reacts preferentially with residues of MIT and DIT located at the surface of the protein, the latter being the major products of slight iodination *in vitro* with ¹²⁵ICl. (Drs. Cahnmann, Toi, and Salvatore.)

Studies on thyroxine formation in synthetic copolymers with polytyrosine regions have

been initiated. These copolymers have a "backbone" of poly-L-lysine, and multiple side chains comprised of L-tyrosine, L-glutamic acid, and D, L-alanine, arranged either as (1)-(ala)_n-(glu)_n-(tyr)_n or (2)-(tyr)_n-(ala)_n-(glu)_n. Although the pK of the phenolic hydroxyl group is lower in (1), the rates of iodination, with ICl, of the tyrosines in (1) and (2) are similar. With a 20% excess of ICl, iodination of (2) is slightly less complete. Reaction of the partially iodinated polymers with DIHPPA is now under study. (Drs. Cahnmann and Sela—Weizmann Institute of Science, Israel.)

The site of tyrosine iodination and thyroxine formation (either by iodination or by coupling with DIHPPA) in proteins of known structure is being investigated in lysozyme, insulin and ribonuclease. In lysozyme, even the tyrosine with pK=12.8 is readily iodinated, and iodination makes this residue more accessible to titration. Thyroxine is produced under special circumstances not yet fully elucidated. In insulin, thyroxine is produced only (or mainly) in the A chain. (Drs. Wolff and Covelli.)

IODOPROTEINS. The structure of the thyroglobulin molecule has been investigated further, particularly with respect to the effects of reduction of the 100 disulfide bridges in the molecule. Complete reduction leads to the formation of polypeptide chains which appear to be $\frac{1}{4}$ the size of the native protein, and have a molecular weight of about 165,000. These are the smallest units obtained thus far without peptide bond splitting. Reoxidation of the completely reduced molecule in air leads to the formation of a protein which closely resembles the native thyroglobulin molecule in sedimentation and viscometric properties, as well as by optical rotary and fluorescence polarization measurements. (Drs. Edelhoch and de Crombrugghe.)

The influence of structure on the iodination of thyroglobulin has been explored. By iodinating the protein in water at pH 7 or in the reduced or non-reduced state in 5M guanidine or 9M urea, it has been found that the structure of the protein is an important determinant of the resulting iodoamino acid distribution. (Drs. Edelhoch and Van Zyl.)

Studies have continued on the heavy thyroidal iodoprotein, the isolation of which was de-

scribed in last year's report. Examination of its physical properties revealed it to be a compact molecule with a sedimentation coefficient ($S_{20,w}^0$) of 27S and a molecular weight of 1,220,000. It had the same amino acid composition and nitrogen content as thyroglobulin (19S) and had identical immunochemical determinants, but the iodine and thyroxine content were higher than that of thyroglobulin in most (but not all) cases. On exposure to low ionic strength, alkaline pH and heat, the 27S protein was shown to be less stable than thyroglobulin and dissociated into 6S, 12S and 19S units. Under mild conditions, however, the 19S unit was the major product. The 27S protein is probably a dimer of thyroglobulin with a somewhat different subunit structure. (Drs. Robbins, Cahnmann, Salvatore, and Vecchio.)

Pulse labeling of the thyroid gland with $^{131}\text{I}^-$ injected shortly before sacrifice was done in rats whose glands had been equilibrium labeled with $^{125}\text{I}^-$ by feeding the latter isotope for 3 or more weeks. The ratio of $^{131}\text{I}/^{125}\text{I}$ was much lower in the 27S protein than in thyroglobulin, indicating a slower rate of iodination of the heavy protein. These studies also revealed ultracentrifugal heterogeneity of thyroglobulin with respect to the two isotopes, indicating a difference between "young" and "old" thyroglobulin molecules. (Drs. Robbins, Salvatore, and Vecchio.)

Iodoproteins were analyzed in the blood and thyroid gland of South African cattle bearing congenital goiter. The animals were given ^{125}I in South Africa, and the frozen materials were shipped to Bethesda. In the blood, the iodoprotein was shown by physical and immunochemical properties to be identical with serum albumin. The serum protein, as in other types of thyroid disease, had been iodinated in the thyroid gland and released into the circulation in large quantity. The iodoprotein in the goiter, however, was very different. It was similar to, but not identical with, thyroglobulin by salting out and electrophoretic analysis, but had a much lower sedimentation rate (~9S). It did not form a precipitate with either antisera to bovine serum or to bovine thyroglobulin, but it did form a soluble complex with anti-bovine thyroglobulin. This appeared to be the first

demonstration of an abnormal thyroglobulin in congenital goiter. (Drs. Robbins and Van Zyl.)

Work has continued on the abnormal soluble iodoproteins found in the tumors and blood of rats bearing transplantable thyroid tumors (Dr. S. Wollman, NCI). In some tumor lines, both tumor and blood contained iodoproteins with the immunochemical properties of serum albumin. In others, however, most of the tumor iodoprotein did not precipitate with anti-rat serum. Further studies are planned to determine whether these tumors produce abnormal thyroglobulin as in the congenital goiter of cattle (Dr. Robbins).

PROTEOLYTIC ENZYMES IN THE THYROID GLAND. A study has been initiated to discover and characterize the proteolytic systems in the thyroid gland which are responsible for thyroglobulin breakdown yielding the hormones, thyroxine and triiodothyronine. Previously described acid proteases are probably not the enzymes responsible for hormone secretion. A particulate thyroid cell fraction, sedimenting between 600 G and 24,000 G, hydrolyzes thyroglobulin over the pH range 7.0 to 10.0. Maximum rates are obtained in the presence of 0.5 to 1.0M salt, which solubilizes the enzyme, and 0.05M β -mercaptoethanol, which reduces the thyroglobulin, making it a better substrate. The products range from free iodoamino acids to peptides with molecular weight of about 100,000. The iodoamino acid yield is increased by a heat-labile supernatant factor, probably a peptidase. After solubilization from cell particles with 1M KCl, the protease has been purified over 200 fold by gel filtration on Sephadex G-100 and DEAE-cellulose chromatography. (Drs. Pastan and Almqvist.)

MEASUREMENT OF IODOCOMPOUNDS IN BIOLOGICAL FLUIDS. Studies have continued on methods for chromatographic resolution of iodide and iodoamino acids on columns of ion exchange resins. Thyroxine and triiodothyronine have been separated using anion exchange resins and organic solvents of high dielectric constant buffered by aqueous solutions of volatile buffers. The pK's of the phenolic hydroxyl groups of thyroxine and triiodothyronine in these solvents were higher than in water, but the ratio of the pK's was approximately the

same. This indicated that differential solvation was important in the separation. The column chromatography procedure is effective in separating milligram quantities of iodide, iodotyrosines and iodothyronines, and can be used with thyroid gland hydrolysates. It has not yet been possible, however, to clarify completely the interference produced by serum. This appears to be due to the thyroxine-binding proteins, and work is currently in progress on this aspect of the problem. (Dr. Lewallen.)

THYROXINE TRANSPORT IN BLOOD. Work has continued on the application of the fluorescence quenching method to the study of thyroid hormone-protein interaction. Results reported last year on the binding of thyroxine to serum albumin have been extended to triiodothyronine (T_3). As in the case of thyroxine, T_3 is bound to a single strong site and several weaker sites. The association constant, however, is weaker in the case of T_3 . (For human serum albumin, $K_{T_4}=1.6 \times 10^6$ moles liter⁻¹ and $K_{T_3}=3.8 \times 10^5$ moles liter⁻¹). These values have been confirmed by standard methods of equilibrium dialysis. (Drs. Robbins, Roth, and Steiner—Naval Medical Research Institute.)

Anterior Pituitary Hormones

GROWTH HORMONE. The properties of bovine growth hormone have been studied by optical rotation, sedimentation, viscosity, fluorescence and fluorescence polarization. A molecular transition occurs in acid (pK=3.55) and also in the neutral pH range. These molecular changes are reversible if ionic strength is maintained at a low level. Further configurational changes have been observed in urea. (Drs. Edelhoch, Condliffe (LNE), and Burger (LNE).)

A specific immunoassay for human growth hormone (HGH) has recently been developed and used to define the plasma HGH level in man and its response to changes in blood glucose. It has already been found that plasma HGH is elevated in acromegaly. The present studies, in which 25 patients have thus far been included, is planned to correlate the abnormalities in HGH function with the usual clinical measurements, to define criteria for

diagnosis, and to determine the response of plasma HGH to pituitary irradiation and estrogen therapy. (Drs. Roth and Glick—Maimonides Hospital.)

Although prolactin and growth hormone are separate hormones in non-primates, attempts to separate them in primates have met with little success. Plasma samples have been assayed for HGH by radioimmunoassay and for prolactin by pigeon crop sac assay. In acromegatics, plasma HGH is high but prolactin is normal. In women 48 hours postpartum, HGH is normal but prolactin is elevated. (Drs. Roth and Bates.)

Presently available assay methods for plasma growth hormone are limited to primates. In order to enable future studies on growth hormone physiology in animals, work is under way to prepare rat growth hormone from over 1000 rat pituitary glands. Antisera which react strongly with monkey growth hormone are also being developed. (Dr. Roth.)

GONADOTROPINS. Studies on gynecomastia in bronchogenic carcinoma have continued. Three men with this syndrome excreted 250 to 50,000 times as much gonadotropic activity in their urine as did eleven control men with bronchogenic carcinoma but no gynecomastia. In two cases available at autopsy, the pituitary gland was depleted of gonadotropin, whereas the tumor contained high titers of gonadotropic activity. Apparently these tumors produced a gonadotropin-like substance which was responsible for the gynecomastia. (Drs. Rosen and Fusco (Mt. Alto V.A. Hospital).)

Further studies have been carried out on the syndromes of hypogonadism and anosmia. At least two distinct syndromes have been recognized. Five patients with the classic syndrome showed decreased urinary gonadotropin (mouse uterus) and no gametogenesis. Patients with variants of the syndrome may excrete normal amounts of urinary gonadotropin, may have gametogenesis, and may have midline cranial defects (cleft lip, etc.). In a kindred of 165 members with a high rate of consanguinity, five cases with hypogonadism, three with anosmia, and six with midline anomalies have been found. Four cases had more than one of these abnormalities. The pedigree was com-

patible with autosomal recessive inheritance. (Dr. Rosen.)

A crude preparation of human pituitary FSH has been further purified by gel filtration on Sephadex G-100 and DEAE-cellulose chromatography. Fractions with 90% or more of the protein in a single band on acrylamide gel electrophoresis have been obtained. Work is under way to obtain antibodies to FSH and to iodinate the purified protein in order to develop an immunoassay for FSH. (Drs. Rosen, Schlaff, and Roth.)

THYROTROPIN. A small quantity of highly purified human thyroid stimulating hormone (TSH) was available for study. This material was assayed in 7 human subjects and compared with bovine TSH. In one subject a dose response curve was obtained. The response was measured by performing 2- or 3-hour thyroid uptakes of ^{132}I , which could be given in multiple doses. No significant difference was found between human and bovine TSH either in the time-course of action or in the dose-response. TSH does not, therefore, share the striking species specificity of growth hormone, and previous estimates of human TSH secretion rate based on response to bovine TSH are confirmed. (Drs. Robbins, Schneider, and Condliffe —LNE.)

Steroid Hormones

Since the last report, the survey of the synthesis of steroid sulfates by the soluble fraction of various bovine tissues has been completed. All tissues synthesized p-nitrophenol sulfate. Testosterone sulfate was formed only by liver. Estrone sulfate was formed by many tissues, and dehydroepiandrosterone sulfate was formed by placenta, liver, adrenal, intestine, pituitary, kidney and uterus. Bovine placenta was much more active than human placenta in the sulfation reaction.

The enzymes which catalyze the transfer of sulfate from adenosine-3'-phosphate-5'-phosphosulfate to hydroxylated steroids are now being purified. There appears to be a family of enzymes specific for the various hydroxyl groups on the steroid nucleus. (Drs. Rosen and Holcenberg.)

Insulin, Glucagon and Carbohydrate Metabolism

ACTION OF INSULIN ON THE LIVER. The inhibition of net protein degradation in the isolated, cyclically perfused rat liver which is produced by insulin *in vitro* has received further study. L-valine-C¹⁴ was used, since this amino acid is presumably not synthesized and only negligible amounts are metabolized in the perfused liver. The steady increase of free valine in the perfusate as a function of time was inhibited 65% by insulin, and valine-C¹⁴ release from previously labeled liver was also inhibited. On the other hand, rates of incorporation of valine into liver and serum protein were unaffected by insulin, nor was there an effect on valine distribution between intra- and extracellular pools nor on release of labeled protein. These findings strongly support the view that insulin acts at a site which normally limits the rate of protein degradation. Specific *inductive* effects on specific enzymes, however, are not excluded. (Drs. Mortimore and Mondon.)

ACTION OF GLUCAGON ON THE LIVER. Glucagon is known to enhance protein catabolism *in vivo* and to stimulate urea formation in the isolated liver. Studies have been initiated using the cyclically perfused liver in order to identify the mechanism of these effects. In the perfused liver, glucagon markedly enhances urea formation but has no effect on total amino acid accumulation. Chromatographic analysis of perfusate amino acids, however, revealed a striking alteration. Glucagon caused a 30 to 40% increase in valine, lysine, leucine and isoleucine, and a 50 to 80% decrease in alanine, glutamate, glutamine and several other amino acids. The urea effect could also be produced by epinephrine and 3',5'-cyclic AMP, but not by 5'-AMP or ATP. The possibility of two effects of glucagon—one on amino acid metabolism *per se*, and another on net protein degradation—is under investigation. (Drs. Mortimore and Mondon.)

GLUCOSE METABOLISM, DIABETES AND HYPOGLYCEMIA. Work is continuing on the kinetic analysis of ¹⁴C-glucose metabolism in man. Utilizing the previously described model, the kinetics of C-6 labeled glucose from the time of its disappearance from blood until its appear-

ance as ¹⁴CO₂ has been analyzed. Two exponentials have been derived for the characterization of the whole body Emden-Meyerhoff pathway. (Drs. Segal, H. Roth, and Berman—OMR.)

Previous studies had demonstrated that glucose inhibits amino acid transport in kidney cortex slices. A study of aminoaciduria in diabetes has, therefore, been initiated. In the small number of patients thus far studied, some had generalized aminoaciduria and one excreted only cystine and lysine. (Drs. Segal and Schwartzman.)

Observations have continued on the use of long acting zinc glucagon in the management of hypoglycemia. One patient with islet cell adenoma, and one with islet cell carcinoma, have been successfully treated for periods of one to two years. Combined treatment with zinc glucagon and adrenal steroid is being explored. (Drs. Segal and H. Roth.)

GALACTOSE METABOLISM AND GALACTOSEMIA. Studies have continued on the possible effects of orotic acid in galactosemia. This substance, a precursor of cofactors important in galactose metabolism, had been reported by others to be effective in enhancing galactose tolerance. As reported last year, orotic acid had no effect on galactose-¹⁴C metabolism in normal or galactosemic subjects. It has also been found to be without effect in protecting rats from galactose-induced cataracts. (Drs. Segal and H. Roth.)

Further studies have been done on the nature of galactose toxicity in newborn rats produced by galactose feeding during pregnancy. In addition to decreased weight of the newborn and of the placenta, cataracts, liver damage and abnormalities of renal tubule cells have been observed. Although histochemical studies indicate markedly reduced liver glycogen, this was not confirmed by chemical analysis. The possible existence of an abnormal form of liver glycogen in galactosemia is suggested. (Drs. Segal and Spatz—Lab. of Histochem.)

It was reported last year that galactose utilization by rat liver decreases markedly with age. Since galactose-1-P uridyl transferase increases, the possible involvement of galactokinase in the age effect was investigated. Galactokinase activity was found to decrease with age

and to parallel the decrease in galactose utilization. Furthermore, a study of the enzyme characteristics (K_m , V_{max} , substrate and product inhibition) indicate that galactokinases in new born and adult liver are different enzymes. (Drs. Segal and Cuatrecasas.)

Amino Acid Transport

Studies on amino acid transport in cystinuria have continued, and a total of 12 patients have been examined. In 10 patients, active transport of both cystine and the dibasic amino acids have been totally absent in intestinal mucosa; in 2 patients, cystine transport was normal but lysine transport was absent. This suggests that two forms of cystinuria exist. In kidney slices, on the other hand, cystine transport was normal in all cases; dibasic amino acid transport was absent. Although cystine and lysine are mutually inhibitory in the intestine, this is not the case in the kidney. (Drs. Segal, Thier and Rosenberg—NCI.)

In further studies on rat kidney, employing Na^+ -free media, various pH's and anaerobiosis, there appear to be distinct transport systems for cystine and for the dibasic amino acids. Furthermore, cystine fails to partake in exchange diffusion with the dibasic amino acids. From these and previously reported findings with human kidney, it can be concluded that human cystinuria is not due to a single defect in renal amino acid transport. (Drs. Segal and Schwartzman.)

Previous reports by others that the animal, genetta tigrina, found in central Africa, has massive cystinuria, prompted investigation of the renal mechanism for this. However, analysis of the animal's urine by ion exchange column chromatography revealed slightly elevated amounts of cystine but large quantities of an unknown amino acid. The latter could be reduced to form cysteine and was very acidic. The compound was identified as S-sulphocysteine by comparing its migration on ion exchange columns, high voltage electrophoresis, and thin layer chromatography, as well as infrared spectroscopy with synthetically prepared S-sulphocysteine. The physiological significance of this amino acid is unknown but it has been shown by others to be found in glu-

tathione as S-sulphoglutathione in rat intestine. (Drs. Segal and Crawhall.)

Protein Synthesis and Protein Structure

PENICILLINASE FORMATION IN *B. LICHENIFORMIS*. Penicillinase, produced as an exoenzyme by *Bacillus Licheniformis*, contains no cysteine or cystine. Mutants requiring cystine for growth were prepared, and penicillinase production was studied in both constitutive and inducible organisms. In both types, penicillinase formation was markedly impaired during cystine deprivation. The absence of preferential synthesis of penicillinase indicates that its synthesis is regulated by the absence of an amino acid which it itself does not contain. (Dr. Segal—at National Institute for Medical Research, Mill Hill.)

CLEAVAGE OF γ -GLOBULIN WITH CYANOGEN BROMIDE. Chemical cleavage of the γ -globulin molecule has not been previously described. Rabbit γ -globulin prepared from antiserum against bovine serum albumin (BSA) was treated with cyanogen bromide (CNBr). This resulted in selective splitting at 6 of the 11 methionine residues in the γ -globulin molecule. Although all methionine bonds could be split by more drastic conditions, this resulted in an insoluble product.

The partial reaction product had a sedimentation coefficient of 5.3S and reacted with anti BSA, retaining all of the activity of the original γ -globulin. The 5.3S fragment also reacted with goat antiserum directed against rabbit γ -globulin or against papain fragment I (Porter) of rabbit γ -globulin, but not against papain fragment III (Porter). CNBr, therefore, appears to cause preferential destruction of fragment III, and this was verified by direct experiment. Reduction of the 5.3S fragment yields a 3.3S fragment resembling papain fragment I. (Drs. Cahnmann, Arnon—Weizmann Inst., and Sela—Weizmann Inst.)

Immunoassay of Peptide Hormones

Immunoassay procedures have opened new horizons in the study of blood levels of the peptide and protein hormones and of the factors which induce changes in these levels. The development of an assay for vasopressin in human

plasma is now being pursued. Arginine vasopressin (AVP) purified from beef pituitary was used to produce antibodies in rabbits and was labeled with ^{131}I . The antibodies are specific for vasopressin and do not react with oxytocin. Some antisera, in fact, can distinguish between arginine and lysine vasopressin. The assay method detects 1.0 m μg of AVP per ml, as yet insufficient for assay of plasma levels. (Drs. Roth, Petersen—NHI, and Klein—NCI.)

CLINICAL HEMATOLOGY BRANCH

Immunology of Blood Cell Deficiencies

NATURE OF THE PLASMA FACTOR RESPONSIBLE FOR IDIOPATHIC THROMBOCYTOPENIC PURPURA. Many investigators have claimed that ITP serum contains an antibody that is detectable *in vitro*, but our work indicated that the positive serologic tests were artifactual. Although ITP sera did not react in immunologic tests *in vitro*, we have found that the platelet-depressing factor in plasma of patients with typical ITP affects autologous as well as homologous platelets, is species specific, is adsorbed by platelets, is present in the 7S gamma globulin fraction of plasma, and produces effects *in vivo* that are quantitatively, as well as qualitatively similar to those produced by known anti-platelet isoantibodies and drug antibodies. These findings leave little doubt that the ITP factor is an antibody. Failure of the ITP factor to react in immunologic tests is not due to its low concentration, but is explicable on the basis of its similarity to known incomplete anti-platelet antibodies.

In this work, new techniques were developed to detect trace amounts of complement components attached to cells, for although gamma globulin was not detectable on platelets exposed to ITP serum, it was conceivable that a reversible antibody reaction resulted in complement-coating of cells. This proved not to be the case, but the anti-non-gamma globulin consumption test that was developed has many applications in the general field of hematology.

Since the only assay for the ITP factor is a biological one, it was necessary to develop methods of fractionating large volumes of plasma to obtain sufficient 7S gamma globulin from ITP plasma for quantitative *in vivo* assay

in man. Commercial methods and methods used by the American Red Cross to prepare gamma globulin from small amounts of plasma (100–500 ml.) through the Cohn fractionation methods that involve alcohol precipitation have resulted in an aggregated product that causes anaphylactoid toxic manifestations. We used DEAE cellulose chromatography to prepare 7S gamma globulin. Our preparation was completely non-toxic when obtained from normal plasma and had only platelet-suppressing activity when obtained from ITP plasma. The DEAE method should have many applications in studies on the *in vivo* effects of human antibodies. (Drs. Shulman, Weinrach and Libre.)

WHOLE BODY COUNTER UTILIZED IN STUDIES OF THE PHYSIOLOGY OF PLATELET PRODUCTION AND DESTRUCTION. Conflicting conclusions have been drawn in a number of different reports in the literature concerning normal and abnormal organ localization of labeled platelets, some implicating the spleen more than the liver and some vice versa. In obtaining our data, we took advantage of the whole body counter developed by Dr. Howard Andrews. In conjunction with him, the counter was calibrated appropriately for organ localization through use of a dummy, the hollow organs of which were filled with solutions containing varying amounts of isotope until conditions observed *in vivo* were duplicated. These experiments established the usefulness of the whole body counter in obtaining more precise information than previously available by any other method for localizing isotopes in specific organs. Normally, when platelets left the circulation, approximately half were found to localize in the spleen and half in the liver. Platelets sensitized lightly by approximately 500 to 800 antibody molecules per cell were sequestered primarily in the spleen, whereas more highly sensitized platelets, containing more than 2000 molecules per cell, were sequestered primarily in the liver. (Drs. Shulman, Weinrach, Libre and Andrews.)

PHYSIOLOGIC BASIS FOR THE EFFECTS OF CORTICOSTEROIDS AND SPLENECTOMY ON CELULAR SEQUESTRATION. It has generally been considered that corticosteroid therapy increases platelet production and thereby has a

beneficial effect in thrombocytopenic disorders. However, it was found that the survival and turnover of platelets in corticosteroid-treated normal individuals and patients was actually normal. Corticosteroids did, however, interfere with splenic sequestration of platelets that were lightly sensitized by antibody. Corticosteroid therapy had relatively minor effects on hepatic sequestration of more heavily sensitized platelets. These findings applied to platelets sensitized by isoantibodies as well as by ITP factor.

That the response to corticosteroid therapy reflects the degree of immune sensitization of platelets and propensity for splenic sequestration was borne out by comparing the effects of corticosteroid therapy and splenectomy with the level of ITP factor in 11 consecutive cases of this disease, all of whom presented with similar low platelet counts and symptomatology. Five of the patients responded well to steroids and had good results from splenectomy, and five had little, if any, response to steroids and poor results from splenectomy. One had a fluctuating response to steroids and splenectomy. The patients who benefitted from therapy had very low plasma titers of ITP factor (less than 1:4), assayed by passive transfer to hematologically normal recipients, whereas patients who did not respond to therapy had high titers of ITP factor (greater than 1:20). The titer of ITP factor determined responsiveness to therapy, for lightly sensitized platelets were sequestered by the spleen and splenic sequestration was inhibited by steroids (and by splenectomy), whereas heavily sensitized platelets were sequestered by the liver as well as the spleen, and this form of sequestration was not inhibited effectively by steroids. (Drs. Shulman, Weinrach and Libre.)

INHIBITION BY RETICULOENDOTHELIAL BLOCKADE OF SEQUESTRATION OF IMMUNOLOGICALLY ALTERED CELLS. In the course of studying the effects of isoantibodies and the ITP factor on sequestration of platelets, some unexpected results were obtained in patients with hereditary spherocytosis whose spleens were approximately 10-fold normal size. It was thought that these patients might show exaggerated responses to factors that sensitize platelets for sequestration, but in fact

their responses were much less than normal to both anti-platelet antibodies and ITP plasma. Since these patients were destroying red cells at 4 to 6 times the normal rate, it seemed likely that excessive red cell destruction in some way interfered with platelet sequestration by the reticuloendothelial system. An opportunity to evaluate the effects of intravenous red cell stroma on thrombocytopenia caused by anti-platelet antibodies was afforded in patients with autoerythrocyte sensitization whom we were attempting to de-sensitize with stroma. It was found that these individuals, when given 1 to 2 grams of red cell stroma intravenously per day, had a diminished response to anti-platelet antibodies and a completely inhibited response when 5 to 9 grams of stroma were given intravenously per day for 4 to 6 days. This is the first demonstration of complete and persistent inhibition of sequestration of sensitized cells through reticuloendothelial blockade. It is especially noteworthy because it was caused by debris of a dissimilar cell type.

Red cell stroma, in the doses used to cause reticuloendothelial blockade, was non-toxic by all of the usual parameters employed in testing new drugs, was easy to prepare from outdated red cells, and convenient to give in a short period of time each day. All other known agents that cause reticuloendothelial blockade, such as thorotrast or gold, are extremely toxic, and do not block sequestration of dissimilar substances. Even aggregated albumin, which has received considerable attention in recent years, produces a very ineffective blockade that lasts for a matter of minutes to hours, rather than days to weeks, and does not inhibit cellular sequestration, as does stroma blockade. Stromal reticuloendothelial blockade has obvious applications in therapy of blood cell immune disorders. (Drs. Shulman, Libre and Weinrach.)

TRANSPLANTATION IMMUNITY. The possible role of blood group isoantigens in homograft and heterograft immunity and the relative importance of classical serologic reactions and delayed cellular hypersensitivity in graft rejection has been found feasible to obtain prolonged graft survival in man. It seems likely from various observations that have been made in man, that homograft or heterograft rejection will not usually be associated with antibod-

ies against the rejected tissue or red cells of the donor that will be measurable by serologic tests available at present. However, there is much information from studies in lower animals indicating that antibodies against leukocyte or platelet isoantigens may be more easily measured and reflect the response of the host to transplantation antigens. Over the past six years we have been identifying monospecific antibodies against genetically determined human leukocyte and platelet isoantigens. We have now identified and have on hand sera against eight specific platelet or leukocyte groups (a minimum of 16 alleles), and antibodies against an equal number of groups that are as yet not completely established as monospecific, but nevertheless can be used effectively in phenotyping cells. These antisera are the only ones available in the world at present for clear-cut typing of individuals in platelet and leukocyte antigen systems. The technique used to establish these antigen systems has been complement fixation. Other techniques that have been used, such as agglutination or antiglobulin consumption, have been totally inadequate for obtaining reproducible results.

Although all of our sera have been obtained from transfused patients who happened to become sensitized, it has been possible to measure inherited isoantigens in other species with these antisera. These isoantigens that were products of genes occurring frequently in the human population were found on cells of all other primates tested, whereas antigens that were products of human genes occurring relatively infrequently were for the most part absent on cells of other primates. No human platelet or leukocyte isoantibodies have been found to react with guinea pig or rat platelets, but one isoantigen reacted with cells of dogs and rabbits, as well as non-human primates.

Of special interest is the fact that three polymorphisms of leukocyte and platelet antigens in man are shared by the higher apes. It has therefore been possible not only to develop antisera against human antigens by injections of human leukocytes and platelets into chimpanzees, but also to identify human polymorphisms by injecting chimpanzee blood into other chimpanzees. Thus it appears that the recent great interest by a number of investigators in ob-

taining human volunteers to undergo experimental and somewhat risky immunization with leukocytes and platelets will not be necessary in view of the interspecies relationships that we have demonstrated.

These investigations have also involved evaluation of antigenic differences between recipients and donors in homografting and in heterografting of man and apes. Our function has been to phenotype animals and man in all recognized platelet and leukocyte antigen systems to help select prospective donors. It is hoped through this cooperative effort that some histocompatibility antigens of significance, both in man and higher apes, will be identified. (This project has been done in association with Drs. Moor-Jankowski of Emory University, Wiener of N. Y. U., Kratochvil of Holloman Air Force Base, Haglin of U. of Minnesota, De-Witt of Tulane U., and Huser of Walter Reed.)

IMMUNOLOGIC THERAPY OF LEUKEMIA. In our studies of isoantibodies against platelets and leukocytes, it was found that specificity could be directed against antigens on platelets only, against antigens shared by platelets and leukocytes, against antigens on both granulocytes and lymphocytes, and sometimes against antigens on either leukocytes or lymphocytes. This high degree of specificity for a cell type permitted producing *in vivo* deficiencies of a particular cell line by passive infusion of antibody in patients with chronic or acute myelogenous or lymphatic leukemia.

We had found by *in vitro* tests that immature, as well as mature cells of a leukemic nature had the same frequency distribution of the recognized leukocyte antigens as did normal leukocytes. The *in vivo* leukopenic effect of isoantibodies in leukemias was in some instances more marked than expected from the effect in normal individuals. This was not due to the greater affinity of leukemic cells for antibody or higher antigen content, for *in vitro* measurements of these parameters were the same for normal and leukemic cells. It appears, however, that there may be a limited reserve of leukemic cells relative to normal cells.

Under some circumstances it appears that isoantibodies will be able to affect bone marrow cells, for we have observed absence of megakaryocytes accompanying isoimmune neonatal

purpura, as well as reduced granulopoiesis in apparent isoimmune neutropenia. Very immature leukocytes that we have been able to test were found to contain the usual concentrations of the recognized leukocyte antigens. It is therefore reasonable to expect that antibodies against leukocyte antigens may be able to affect bone marrow precursors of malignant cells and exploration of this possibility is at present underway. (Dr. Shulman.)

DRUG SENSITIZATION. It has generally been accepted that once an individual becomes sensitized to a drug, as in drug purpura or drug hemolytic anemia, that he never again will be able to take the provocative drug. However, we found that three patients who had had drug purpura and high titer drug antibody did not develop significant antibody levels on challenge with the drug after decay of their initial antibody. This is the first observation in man or animals of apparent immune tolerance to haptenic antigens. Our current investigations of this problem involve tissue culture and other *in vitro* techniques that may detect a form of drug antibody not detectable by conventional serologic tests. (Drs. Shulman and Libre.)

ISOIMMUNE NEONATAL PURPURA. Neonatal purpura occurs about once in 10,000 births, is frequently benign and self-limited, but fatal in approximately 20% of the severely affected infants. If the mother is hematologically normal, the most likely basis for purpura is isoimmunization of the mother by fetal platelets and transplacental transfer of antibody to the fetus. Physicians throughout the world have sent us blood samples on families in which isoimmune neonatal thrombocytopenia is suspected. Platelets from these family groups are phenotyped and maternal sera are tested for antibody. We have been able to do these analyses by mail because the complement fixation system that we use does not require fresh cells and sufficient platelets are obtained from a 20 ml. sample of shipped blood for phenotyping with the 12 monospecific isoantibodies that are available. Over 150 families have been studied in this way, 30 of them on more than one occasion.

The frequency of immunization by particular isoantigens has provided unique information concerning antigenicity of platelet anti-

gens. Although the PI^{A1} antigen is mismatched in only 2% of pregnancies, it accounts for more than 50% of the cases of isoimmune neonatal purpura, hence is the most antigenic antigen recognized so far. The next most potent antigen is one shared by platelets, granulocytes and lymphocytes, labeled PIGrLy^{B1}. This accounts for about 1/4 of the detected antibodies. Information on antigenicity is pertinent not only to the problem of neonatal purpura, but obviously also to the problem of transplantation immunity.

This work has also been aimed at assessing better forms of therapy in neonatal purpura. In collaboration with many of the physicians who have sent us samples, serial observations are being made on repeated births in single families to determine the efficacy of steroids or exchange transfusion with respect to titer of maternal antibody and clinical findings shortly after birth. So far it appears that steroids to the mother antenatally may benefit the child during birth, but that exchange transfusion is the only reliable form of therapy in most severely affected infants. It has been possible to predict, so far with complete accuracy, the occurrence, as well as the nonoccurrence, of isoimmune purpura in subsequent children in 30 families in whom an initial case of isoimmune purpura was documented. (Dr. Shulman.)

MISCELLANEOUS. The clinical material that we have seen has resulted in studies on several patients who, although not falling into a particular research category, had notable clinical disorders worthy of report. One of these patients represents the first documented case of myocarditis due to a PPLO organism identified by serology and culture in association with Dr. Chanock. Other cases were rare erythrocyte abnormalities, some associated with chromosomal aberrations, studied with Drs. Brecher and Wang. (Drs. Libre, Weinrach and Shulman.)

Blood Coagulation and Diseases of Hemorrhage and Thrombosis

SURGERY IN HEMOPHILIACS. Our previous studies on the effectiveness of commercially available Fraction I in surgery of hemophilia has been extended to include an additional three patients. One of the major contribu-

tions of this study is establishment of a regimen of Fraction I therapy that does not require laboratory control. It was based on *in vitro* determination of the potency of the Fraction I concentrate and our previous work that established the intravascular decay rate and body distribution of Factor VIII. Our patients were managed in the pre- and post-operative period by estimating their requirements of Factor VIII based on these parameters. The amounts used proved to be totally effective and efficient without requiring laboratory assay of blood level, a circumstance that will permit treatment of hemophiliacs in hospitals that do not have research coagulation laboratories. (Drs. Shulman and Marder.)

DENTAL EXTRACTIONS IN HEMOPHILIACS. This special area of hemophilic care was investigated in association with NIDR. Although much has been written about the importance of local care after extractions (i.e., approximation of tissue, dental splints, socket pads, etc.), we found the only reliable means of preventing and controlling bleeding after major dental surgery was Factor VIII therapy with Fraction I. Amounts necessary for proper hemostasis were sometimes greater than those required for major abdominal surgery and the proper timing of Factor VIII infusions differed after dental compared to nondental surgery. We have established a satisfactory regimen of Factor VIII therapy to cover dental surgery of all types that can be followed without recourse to laboratory control. (Drs. Shulman and Marder and Dr. Gamble, NIDR.)

HEMORRHAGIC MANIFESTATIONS OF ALDRICH SYNDROME. Aldrich syndrome is an X-linked inherited disease in which male children are affected with eczema, aberrations in gamma globulin synthesis and thrombocytopenic purpura. It has generally been considered that the thrombocytopenia of this syndrome is immune in nature because of the associated abnormalities in gamma globulin. However, it was found by studies of three cases, that platelets survive normally and that hemorrhagic manifestations can be controlled for the normal period of platelet survival (on the order of 8 to 10 days) by a single platelet transfusion. The chief abnormality noted in these patients was not a deficiency of megakaryocytes, but a peculiarity in

their maturation, with evidence of nuclear degeneration and over-maturation out of phase with cytoplasmic platelet production. It appears that an abnormality in megakaryocyte function may underlie the hemorrhagic manifestations of this disease. There is no other known disorder in which this type of abnormality has been documented. (Drs. Shulman, Pearson, U. of Florida, and Oski, U. of Penn.)

THROMBOCYTOPENIA OF INFECTIOUS DISEASES. Studies on normal controls in NIAID carried out approximately two years ago show that thrombocytopenia invariably develops during the course of initial parasitemia in experimentally induced vivax malaria. A similar thrombocytopenia was observed to occur by Sodeman and Jeffery in malaria induced by a simian strain in Rhesus monkeys. We have attempted to define the basis for thrombocytopenia in this disease. In order to do this, the life span of platelets in normal monkeys has been determined and a very simple approach to estimating bone marrow function in Rhesus Monkeys was developed. It is essentially an isotope dilution technique using Cr⁵¹-labeled platelets.

Thrombocytopenia was closely correlated with the degree of parasitemia. A marked diminution in the life span of platelets occurred, and in some animals slight inhibition of megakaryocyte function was also discernible. Since most previous studies have suggested that bone marrow inhibition is the primary basis for thrombocytopenia associated with infectious diseases, this is a significant finding that is currently being further evaluated to determine whether a humoral factor related to anti-malarial antibodies, or conceivably a thromboplastic component related to tissue degradation, underlies the shortened survival. (Dr. Shulman and Drs. Sheagren, Sodeman and Jeffery of NIAID.)

In attempting to develop an animal model for the thrombocytopenia that develops in association with rubella and other exanthems, ferrets, the only known animals susceptible to rubella, were infected with the same strain of virus that produced thrombocytopenia in man experimentally. The ferrets, however, did not develop thrombocytopenia, despite the fact that they developed viremia. Although no positive

findings resulted from this, the pitfalls of translating animal experimentation to man in this area of investigation were clearly demonstrated. (Dr. Shulman and Dr. Sever of NIAID.)

There is general agreement that certain bacterial infections, particularly septicemia with gram-negative organisms, results in fulminant thrombocytopenia. The basis for this is obscure, although it has been suggested that the endotoxins of gram-negative bacteria may induce thrombocytopenia in a manner similar to that observed in rabbits following injections of pyrogenic endotoxin. It was found that Rhesus monkeys and dogs are completely resistant to the thrombocytopenic effects of endotoxin, in contrast to the marked susceptibility of rabbits. Moreover, Wolff and associates had found that man was resistant to the thrombocytopenic effects of endotoxin in amounts sufficient to produce febrile responses. The susceptibility of rabbits appears to be related to their unique ability to temporarily sequester platelets, probably in their spleens, in contrast to the lack of this ability in primates and some other animals. Studies are continuing of the dynamics of platelet sequestration in rabbits. (Dr. Shulman and Drs. Wolff and Sheagren of NIAID.)

CONTINUED EVALUATION OF AN UNUSUAL FORM OF PAINFUL PURPURA. During the past year we have seen an additional three cases of autoerythrocyte sensitization, making a total number of 14 patients observed here. This peculiar syndrome which occurs only in women and is associated with reactions resembling delayed sensitivity when erythrocytes are injected intradermally, has many of the characteristics of an immune disease, yet has defied attempts to document this. In trying to find some common basis for the skin manifestations, an interesting lead has arisen in that almost all patients have required treatment for urinary tract infections due to coliform bacilli. A polysaccharide separated from the culture filtrate of *E. coli* obtained from the urine of one such patient has produced positive skin tests in the patient herself, and in another case of autoerythrocyte sensitization. Since this polysaccharide resembles blood group substances structurally, it is conceivable that a cross-reaction of

an antibacterial antibody with an erythrocyte component may be the cause of the skin reaction. (Drs. Shulman, Libre and Weinrach.)

VON WILLEBRAND'S DISEASE. We have continued our studies of von Willebrand's disease, an unusual form of hemophilia associated with prolonged bleeding time due to lack of a plasma factor that is necessary for vascular integrity, plus frequently an associated low level of Factor VIII, the factor that is deficient in classical hemophilia. In contrast to the classical form of Factor VIII deficiency, von Willebrand's disease is not sex-linked and is dominant rather than recessive. Moreover, von Willebrand patients show a remarkable rise in Factor VIII after infusion of plasma from classical hemophiliacs who totally lack Factor VIII on the basis of *in vitro* assays. It therefore appears that plasma of patients with classical hemophilia contains an inactive precursor of Factor VIII and suggests that the genetic abnormality in classical hemophilia may be an abnormality in an operator gene, rather than a complete deletion. There is hope therefore that therapy of hemophiliacs may be approached through activating a precursor, rather than by replacement therapy. Kinetic studies of the activation of this precursor in patients with von Willebrand's disease that are currently underway may provide insight into methods of activating Factor VIII *in vitro* and possibly *in vivo*. (Drs. Shulman and Libre.)

METABOLIC DISEASES BRANCH

Mineral Metabolism

Clinical Investigations of Normal and Abnormal Bone Metabolism

The primary research emphasis has continued to be on the interrelationships between dietary calcium and various nutritional factors and the pathogenesis and treatment of demineralizing diseases. The factors controlling the absorption of dietary calcium and techniques for measuring absorption were particularly investigated in this period.

GASTROINTESTINAL ABSORPTION OF CALCIUM. Last year development of a new technique for measurement of absorption of calcium was reported based on the oral admin-

istration of calcium-47 and rate of appearance and level of radioactivity attained in an extremity (arm). During the past year the procedure has been so extensively modified as to amount to an entirely new approach. The new technique measures net transfer of calcium from the lumen of the gastrointestinal tract into the blood stream and has permitted evaluation of calcium absorption in normal individuals and in patients with metabolic bone disease. The technique is based on the mathematical derivation of a transfer function from data obtained by sampling the radioactivity in one or more compartments after separate oral and intravenous administration of calcium-47. Following intravenous administration of the radioisotope, blood samples are drawn frequently during the first 6 hours and then daily for 6 days for counting in a well type spectrometer. One arm is also counted at frequent intervals during the same time period in the Armac, a large volume liquid scintillation counter. After approximately one week, the same procedure is repeated following oral administration of calcium-47. Urine and stools are collected and analyzed for radioactivity and stable calcium. Transfer functions (signifying the rate of absorption and the percent of the administered dose absorbed for any time period) are generated mathematically using the blood and arm data separately, and these results are compared to net absorption calculated from stool radioactivity. Agreement of the data from the three modes of calculation will permit the elimination of blood and stool collection, and allow reliable transfer rates to be calculated from arm counting alone.

Preliminary observations indicate good agreement between the three values, with a normal range of net calcium transport from 35%–55% of an administered dose. In addition, these observations present the first demonstration of actual absorption curves. The rate of calcium absorption is most rapid during the first 30–45 minutes following oral administration. For any given test dose, the bulk of calcium absorption is completed by 6 hours. Although most patients with osteoporosis seem to have normal calcium absorption, one patient with severe and progressive osteoporosis has

definite calcium malabsorption in the presence of absorption tests for other constituents that are within normal limits (fat, glucose, d-xylose). Definite subnormal absorption (10%) has been demonstrated in a patient with non-tropical sprue who has subsequently been placed on a gluten-free diet, for studies first without and then with vitamin D administration.

In addition to providing useful clinical information about calcium absorption which may be of therapeutic benefit to patients with disorders of mineral metabolism, these studies will permit precise investigation of the process of calcium absorption *in vivo*, since the actual rate of calcium absorption is determined for any given time. [Drs. Peck, Birge, Whedon, Berman and Barondes.]

Connective Tissue Metabolism in Bone and Cartilage

INHIBITION OF COLLAGEN SYNTHESIS IN SURVIVING CALVARIA FROM FLUORIDE TREATED RATS. Stimulation of bone formation by fluoride ion has been suggested by the presence of increased bone density in accidental and experimental skeletal fluorosis, increased calcium retention in some patients with osteoporosis and Paget's disease treated with fluoride ion, and evidence from x-ray diffraction and calcium isotope exchange studies of fluorotic bone. Despite these observations, the *in vivo* effect of fluoride ion on metabolism of the organic matrix of bone has not been evaluated. For this reason, bone collagen synthesis was studied in surviving calvaria and in cartilage segments from growing rats treated with 10–50 PPM fluoride ion in the drinking water for 2 weeks to 10 months. Collagen synthesis was evaluated by the *in vitro* conversion of free L-proline U-C-14 to peptide hydroxyproline C-14, a process unique to collagen formation in mammalian tissues. Significant inhibition (34–53%) of both unaggregated soluble and aggregated insoluble forms of collagen was produced by treatment of rats with 50 PPM-F for 2 weeks, 1 month, 6 months and with 10 PPM-F for 10 months, while 10 PPM for 1 month had no effect. In addition, small (13%) but significant ($p = <.01$) reduction of total collagen content

was found after 50 PPM-F for 1 month. These results correlate directly with the skeletal fluoride concentration. Preliminary experiments have indicated no inhibition of collagen synthesis in *cartilage* segments from fluoride rats while the expected inhibition was found in calvaria from the same animals.

These results indicate that reduction of collagen synthesis in calvaria from fluoride treated rats is related to the high skeletal concentration of fluoride ion. Observations on proline pool size and free amino acid transport by fluorotic bone are now being carried out. Additional studies include calcium and phosphorus content, ash weight, water content and specific density determinations on selected bone. [Drs. Peck, Zipkin (NIDR) and Whedon.]

BONE CELLS: BIOLOGICAL AND BIOCHEMICAL OBSERVATIONS FOLLOWING ENZYMATIC ISOLATION. Development by this unit of a new technique for harvesting large numbers of viable bone cells by digesting fetal rat calvaria with collagenase *in vitro* has presented an unique opportunity to study bone metabolism at the cellular level without the limitation of a dense connective tissue barrier. Cells harvested after bone digestion were found to be histologically intact, viable as indicated by vital dye accumulation and multiplication in cell culture, and to be similar to whole bone segments in patterns of glucose metabolism. A large percentage of isolated cells contained intense alkaline phosphatase activity.

Initial studies have been designed to evaluate differentiation of bone cells and their response to humoral and non-humoral substances. A technique for studying collagen synthesis was developed using the formation of collagen-specific labeled hydroxyproline from labeled precursors, followed by sonification of cells, separation and isolation of protein on Sephadex G-25 columns, hydrolysis and chromatography. Collagen synthesis is linear until 12-16 hours after isolation, and no collagen synthesis can be detected when isotope precursor is added after 24 hours preincubation in suspension culture. Collagen synthesis is enhanced by the presence of dialyzed serum albumin in the incubation mixture, and also by the presence of complex culture media such as Eagle's #2. Synthesis is inhibited by sodium fluoride and

by insulin. In addition, isolated cells actively transport alpha-amino-isobutyric acid, a process inhibited by fluoride and iodoacetate. Further studies of the effect of fluoride ion on transport, protein synthesis and glucose metabolism by isolated bone cells are in progress. [Drs. Peck and Birge.]

Energy Metabolism

Studies of human energy metabolism involving use of the Metabolic Chamber for continuous, long term analysis of respiratory oxygen and carbon dioxide exchange are concerned with a number of physiological and metabolic problems. These investigations deal with disordered metabolism in immobilization, effects of various agents on fat mobilization and oxidation, and mechanisms of temperature regulation as affected by hormonal action and in various diseases. Collaboration with increasing numbers of groups in this and in other institutes is now a prominent characteristic of the activities of the Metabolic Chamber staff.

Physiologic Studies of Immobilization

In order to evaluate certain metabolic alterations (in addition to those previously studied) that occur during prolonged periods of very limited physical activity such as are presented in certain clinical conditions, such as coronary thrombosis, post-accident, etc. and those that will be found in space travel, a limited study of immobilization was undertaken. Thus far two normal young men have been confined to bed for periods of two and four weeks. Prior to the confinement, during it, and through a recovery phase, a series of measurements were made. Immobilization consisted of complete bed rest with the subject using the bed pan, receiving bed baths and being lifted to the balance for weighing and to the tilt table for the measurement of vascular response. The subject was allowed to feed himself and to turn the pages of a book. He was maintained in a Gatch position with his head and upper body elevated 45° and his knees flexed.

Energy metabolism: The metabolic measurements included oxygen uptake and carbon dioxide production during a regular course of four levels of physical activity; basal, a hand-eye

coordination activity, a muscle power activity using the feet, and a period of rest during which a high protein meal was digested. Comparison of the metabolic rates in the pre-immobilization, immobilization and post-immobilization phases for each activity level showed no significant changes.

Metabolic responses to intravenous glucose and insulin: Abnormal responses to the intravenous injection of glucose and insulin have been reported in obese subjects by this group. Similar studies were done on the two normal subjects of the immobilization study during the three phases of the experiment. Evidence was strongly suggestive, but not conclusive, that the same patterns seen in the obese subjects (lessened response to intravenous insulin and delayed response to glucose loading) were seen in these subjects during their immobilization phase.

Physiological responses during immobilization: During the three phases of the study, before, during and after immobilization, various indices of physiologic function were followed, including serum chemistries, renal function, caloric expenditure, urinary steroids, calcium balance, and orthostatic vascular response, in addition to the special studies mentioned above.

As in previously reported studies, the subjects developed marked hypercalciuria and negative calcium balance during the immobilization phase. Urinary steroids, both 17-keto- and 17-hydroxy-, were observed to increase during immobilization. With the use of a tilt table, changes in pulse pressure and pulse rate were studied following the change in inclination of the patient from the horizontal to 60°. The anticipated narrowing of the pulse pressure was observed after 25 minutes at 60°, and this response became progressively more pronounced during immobilization. Similarly, the increase in pulse rate on tilting observed during immobilization showed an increase over that of the pre- and post-immobilization periods. [Drs. Birge, Thompson, Peck and Whedon.]

Physiological Studies of Temperature Regulation

THERMOREGULATION AGAINST COLD IN NORMAL SUBJECTS AND IN PATIENTS WITH

RECURRENT FEVER. Investigations of thermoregulatory responses to cold air are being continued in normal subjects to extend base-line information for similar studies also being conducted with patients with various diseases. As described in earlier reports, the insulating effect of subcutaneous body fat and total body fat in moderating the impact of cold on the body has been partially but not completely characterized. It is apparent, however, that body fatness must be taken into account to assess properly the thermoregulatory responses in patients. Eleven patients with recurrent fever and four with experimentally induced malaria have been exposed to cold (50° F), each on two or more occasions. Although the thermoregulatory responses frequently fell within the normal range established by earlier Metabolic Chamber work, the following findings suggest possible abnormalities in the reaction to cold in patients with recurrent fever:

- (1) Heat production was frequently greater than would have been predicted from studies on normal subjects of comparable body fatness.
- (2) Deep body temperatures (ear, esophageal or rectal) frequently fell immediately and more rapidly following exposure to cold in contrast to the early elevation (0.1–0.3° C) in core temperature usually seen in normals (indicating a defect in heat conservation physiological mechanisms).
- (3) Cold exposure following experimental malaria induced, in one patient, elevated heat production, greater peripheral heat loss, and more rapid core cooling than seen in control experiments performed in this man after administration of malarial parasites but before malarial chills and fever became evident.

These studies indicate that long-standing recurrent fever and experimental malaria may, in some patients, disturb temperature regulation against cold to a measurable extent and that further exploration of the nature and extent of these abnormalities would be worth-

while, as potentially applicable to improved therapeutic management of febrile illnesses. [Dr. Thompson with Dr. Wolff of NIAID.]

THERMOREGULATION AGAINST COLD IN PATIENTS WITH NEUROLOGIC BRAIN DISEASE. Studies were conducted in two patients with pinealoma as part of a continuing study on the relative influence on thermoregulatory capacity of various types and degrees of brain lesions and severe brain disease. In one case, 4 weeks before death, the patient's core temperature continued to fall in the cold to levels near 35° C without any active peripheral responses to cold and no indication of elevating metabolic rate. In a less severe case, peripheral vasoconstriction appeared to be more normal but response of heat production by elevation in metabolic rate and by shivering did not occur until the rectal and ear temperatures had fallen to 36° C. [Dr. Thompson with Dr. Wolff of NIAID and Dr. Ommaya of NINDB.]

Energy Metabolism during Alterations in Endocrine Function

BASAL METABOLIC RATE AND RESPIRATORY QUOTIENT IN PATIENT WITH ACROMEGALY. Oxygen consumption and carbon dioxide production studies on a series of patients with acromegaly have been initiated, rates being measured prior to and following therapy. Thus far, contrary to earlier reports, the basal metabolic rate appears to be essentially normal in these individuals with increased pituitary growth hormone activity. [Drs. Thompson and Roth.]

METABOLIC RATE ALTERATIONS DURING EXPERIMENTALLY INDUCED CHANGES IN ENDOCRINE HORMONE LEVELS. The Metabolic Chamber has been utilized to study the relationship of the thyroid-pituitary axis to free thyroxine blood levels. Enovid has been administered to normal volunteer subjects to provide a large number of individuals with an induced rise in serum thyroxine-binding-globulin (TBG). Studies thus far suggest that free thyroxine falls at TBG rises, but when equilibrium is achieved after Enovid administration, although the percent of free thyroxine remains depressed, the computed total free thyroxine

level rises to normal values. Efforts to identify the role of TSH in this response are planned.

The interactions between thyroxine-binding-globulin and thyroxine in the regulation of pituitary-thyroid function under conditions of environmental temperature change are being studied. The observation has been made that when normal volunteers are exposed to temperatures of 50° F the serum PBI and free thyroxine rise by 25–50% within two hours. The traditional explanation of *heat* induced thyroid inhibition has been that this is accomplished through changes in thermoreceptors, or through depression of the requirement for thyroxine. The present observation in the opposite climatic situation suggests an alternative mechanism. A dissociation of thyroxine from binding sites in tissues and from thyroxine-binding-globulin might occur which could inhibit the pituitary-thyroid axis.

METABOLIC RATE IN VARIOUS DISEASES OF ANIMALS. Studies aimed at elucidating the nature of the anemia of hypothyroidism in the dog have been initiated in collaboration with the National Cancer Institute. Based on the principle and general instrumentation of the Metabolic Chamber, a metabolic chamber suitable for determination of the 24-hour metabolic rate in an unencumbered dog has been designed. Previous metabolic measurements on restrained dogs have been highly variable, but after a period of training in the new cage-size chamber, normal resting dogs show reproducible and expected metabolic rates over a 24-hour period. Twelve normal dogs had values of 36.7–47.3 kcal/kgm. per day, with a mean of 42.8. Thirteen hypothyroid dogs had metabolic rates of 21.4–29.6 kcal/kgm. per day, with a mean of 24.8. Four hypothyroid dogs treated for seven weeks with .06 mgm/kgm. of levothyroxine administered parenterally in a single daily dose showed return to normal and super-normal levels in metabolic rate and a return of red cell volume to normal within seven weeks. Thus far, administration of alpha-2,4-di-nitrophenol in doses sufficient to raise the mean 24-hour metabolic rate to twice the normal level failed to return red cell volume toward normal. [Dr. Thompson, with Drs. Hollander, Barrett, and Berlin, NCI.]

Electrolyte Balance in Heat Stress

Although the role of heat stress and sweating in the production of sodium depletion and consequent heat exhaustion has long been known, the possibility that these factors might affect the balance of other electrolytes has received less attention. A study of the effects of heat stress on potassium balance, initiated in the National Heart Institute, has been transferred to NIAMD as of December 1964. In this investigation, normal volunteer inpatients are studied on a diet comparable to the common diets of the countries of Southeast Asia, and subjected to moderate heat stress. Their potassium balance is followed by means of the Whole Body Counter, and these data correlated with potassium intake and output observed by traditional balance techniques. By difference, the overall effect of sweating in causing potassium loss can be estimated.

So far, five normal young men have been studied. Results on the first two, reported in April to the meeting of the American Physiological Society, showed cumulative potassium losses of approximately 7% of initial potassium content, with electrocardiographic abnormalities developing within 5 weeks. No heat exhaustion or other symptoms attributable to potassium depletion were noted. The potassium concentration in sweat did not appear to fall sharply as depletion developed, indicating that the sweat glands do not have an effective mechanism for preventing further potassium losses.

Study of the second group of three volunteers is still in progress. These men have been on the potassium-depleting regimen for a longer period, and, as might be expected, the overall losses have been greater. Data on sweat potassium concentrations are not yet available.

Since the conditions employed in this study are closely comparable to those that affect large populations in tropical countries, as well as United States personnel serving in these areas, the initial finding that potassium equilibrium is easily upset requires further investigation. Possible interrelationships between potassium balance under heat stress and other nutritional and endocrine factors (such as magnesium balance, on the one hand, and

adrenal mineralocorticoid secretion, on the other) must be clarified. The possibility that potassium depletion plays a causative role in certain diseases of hot climates makes further study along these lines imperative. [Drs. Gordon, Thompson, Birge, Andrews (C.C.) and Cage (NCI).]

PEDIATRIC METABOLISM BRANCH

In the past year the investigations conducted in this Branch have concentrated on cystic fibrosis of the pancreas in an effort to elucidate the pathogenesis of this inborn error of metabolism. Three principal lines of study have been followed:

1. An attempt to uncover and define by immunochemical techniques the defect of mucopolysaccharide metabolism thought to be responsible for the protean manifestations of this disease.

2. A study of the physio-pathologic mechanisms responsible for the striking and unique abnormality of sweat and other body fluids in cystic fibrosis.

3. Other clinical and pathologic studies aimed at improving the knowledge of the pathogenesis, course and complications of cystic fibrosis and improving its treatment.

Immunochemistry

PSEUDOMONAS AERUGINOSA AND ITS SLIME IN RELATION TO THE PATHOGENESIS OF CYSTIC FIBROSIS. In the search for the biochemical defect underlying cystic fibrosis, our attention was turned to certain unusual and possibly unique findings in the bacterial flora of patients with this disease. *Pseudomonas aeruginosa* is of special interest since this organism when cultured from patients with cystic fibrosis commonly undergoes a mucoid degeneration and produces large quantities of viscous slime, to a degree apparently unparalleled by any other human pathologic condition. This may not only aggravate the patient's condition, but more importantly may give a clue as to the basic underlying defect in cystic fibrosis. There indeed is reason to believe that this change may be induced by a chemical compound produced by fibrocystic patients which acts as a substrate.

Many questions, therefore, arise as to the relation between the host and the parasite, the

chemical composition of this slime and its specificity when obtained from organisms cultured from fibrocystic patients, the role it plays in the chronic inflammatory and obstructive pulmonary disease, and finally the possibility of inhibiting its production or decrease its viscosity by chemical and enzymatic means.

Electron-microscopy of these slime producing organisms demonstrated long, slightly branched filaments scattered around each organism, but not symmetrically attached to the organism itself. Analytical ultracentrifugation demonstrated that the slime material is very dependent on concentration behaving like a long polymer. By paper chromatography the major components of this material were identified as mannuronic acid and mannuronolactone. Antibodies against this extra-cellular bacterial product were demonstrated in many of the sera from patients with cystic fibrosis from whom mucoid strains were isolated. Only a small number of fibrocystic patients with non-mucoid *Pseudomonas* had similar antibodies which were completely absent in 55 control sera from other patients at the NIH.

These findings have created interest in many laboratories throughout the country, and the answers to the many other questions raised in the course of these investigations are being sought.

SEARCH FOR THE INBORN ERROR OF MUCOPOLYSACCHARIDE METABOLISM IN CYSTIC FIBROSIS BY IMMUNOLOGIC AND BIOCHEMICAL METHODS. It is thought that cystic fibrosis is the result of an inborn error of mucopolysaccharide metabolism, but the basic defect in this generalized disease is still not known. If a unique antigenic mucoprotein were detected, this would provide a tool for further genetic studies and for direct investigation of biochemical lesions.

Accordingly immune antibodies were produced in rabbits and in donkeys by inoculation of macromolecular mixtures from urine, saliva, pancreatic cyst fluid, and tissue homogenates from four organs. In all studies, material from normal controls and patients with cystic fibrosis were compared. Immunoelectrophoresis and micro-immunodiffusion techniques were used to detect specific differences between macromolecules of normals and fibrocystic sub-

jects and to follow the effectiveness of chemical separation and purification. Samples of body fluids from CFP patients were subjected to chemical separation and analysis by a variety of methods (e.g.: continuous flow electrophoresis, column chromatography, starch-gel electrophoresis, sephadex-gel filtration, acrylamide-gel electrophoresis, and salt fractionation). Analytical ultracentrifugation was performed of the urinary mucoprotein of Tamm and Horsfall and its fractionated subunits.

Patient material was obtained from our wards and outpatient facilities and through the cooperation of Children's Hospital. Tissues were collected from autopsy material from patients with CFP at the NIH. Tissues from normal children killed in traffic accidents were obtained as controls.

a. *Study of urinary macromolecules.* Previous investigations were continued in some instances completed and reported. No specific antigenic determinants in the urine of patients with cystic fibrosis have been found, and there was good immunologic identity of various fractions in the two groups compared even after chemical fractionation. On analysis some chemical differences between CFP patients and normal children controls were present, however, their significance is doubtful until much further work is done.

The absence of the arc of transferrin in immunoelectrophoresis in most CFP urine macromolecular concentrates reported last year has now been confirmed using acrylamide-gel electrophoresis. The total iron binding capacity and autoradiography studies after incubation with Fe-59 and serial dilution showed no significant difference between CFP patients and controls. However, specific estimation of serum transferrin levels by quantitative immunodiffusion revealed low serum transferrin in three of four patients studied. Low serum levels may very well explain the absence of this mucoprotein in the urine of CFP patients.

Two albumin components have been found in the urine of several patients with CFP. Although immunologically identical, these albumin moieties have different mobility in acrylamide-gel electrophoresis. The significance of the two albumin components is unknown at present; however, it is not found in all urines from

CFP patients and seems to be associated with severity of this disease. Both components can be recovered from acrylamide-gel in quantities which render chemical characterization possible.

b. Tamm-Horsfall (TH) Urinary Mucoprotein. The TH mucoprotein was thought to be of special interest, because a physico-chemical abnormality has been shown to exist in fibrocystic patients by Maxfield. The genetic significance of this mucoprotein was further pointed out by Keutel in Germany, who reported its absence from the urine of Negroes, a group in which cystic fibrosis is rare.

White and Negro children with CFP, as well as white and Negro normal control children were extensively studied. Specific immune serum against the TH mucoprotein was prepared in our laboratory, and further studies of this urinary component have been initiated with respect to its physical characteristics (e.g.: sedimentation, diffusion, viscosity). Subunits of the TH mucoprotein were also prepared and studied.

It can be concluded that the TH urinary mucoprotein is immunologically identical in patients with CFP and controls. It can also be stated that the American Negro children with CFP that have been studied have TH mucoprotein antigenically indistinguishable from that of normal Negro and white children. Attempts to obtain urine from an African Negro population will be undertaken to confirm or deny the finding or absence of TH mucoprotein in urine of Negroes without admixture of white blood.

SEROLOGIC REACTIONS IN CYSTIC FIBROSIS. This investigation was undertaken as part of the study of host defense and host response to antigenic challenge in cystic fibrosis.

28 sera of CFP patients and 27 sera from normal controls were tested for immunoglobulin levels. The IgG in the cystic group were elevated and differed significantly from the normals with a value for $p < 0.01$. For IgA the cystic group was also significantly elevated with a $p < 0.001$. Preliminary results suggest that the IgM immunoglobulins are not elevated in the cystic fibrosis group tested.

There was special interest in antibodies against chloramphenicol because of the pre-

viously reported optic neuritis occurring in 6 of our patients treated with this drug. However, we were unable to detect antibody to the chloramphenicol-albumin conjugate when sera from 8 cystic patients treated with chloramphenicol (one had a history of optic neuritis presumably due to this drug) were used as a source of potential antibody. In only one of 27 sera there were antibodies against penicillin.

SALIVARY SECRETION OF ABH BLOOD GROUP SUBSTANCES IN CYSTIC FIBROSIS. These studies were undertaken as part of the immunologic studies and in order to elucidate the genetic background of cystic fibrosis, which is poorly known. The aim of the investigation was to study a possible genetic linkage between ABH secretion status and cystic fibrosis.

49 (76.9%) of the CFP patients were secretors and 16 (23.1 \pm 5.3%) were non-secretors. 31 (77%) of the controls were secretors and 9 (23%) non-secretors. These frequencies did not differ from those reported for normal population: 76–78% secretors and 22–24% non-secretors. No linkage was demonstrated.

Effects of Aldosterone, Other Steroids and Diuretics on Sodium Transport in Sweat Glands and Kidneys in Patients with CFP and Normal Controls:

Previous investigations of the effect of aldosterone on sodium transport in sweat glands and kidneys have been continued and extended. The same basic protocol was followed in all of these studies both for control subjects and cystic fibrosis patients. To all subjects, aldosterone in sesame oil was administered every 8 hours I.M. in a constant dosage. Sweat was obtained by pilocarpine iontophoresis.

NORMAL CONTROLS. Previously reported studies on adult males were extended to include normal adult females and normal children. Normal adult females responded to endogenous aldosterone administration in exactly the same manner as adult males. As in the cases of the males, the sweat of females as compared to their kidney response was very different in onset and duration, although quantitatively similar.

Quite surprising were the findings in 6 normal children ages 9 to 12 years. While the sweat response was comparable to that of young adult group studied, the renal response was quite different. No abrupt fall in urine sodium occurred, no step-wise "escape" was found and the urine sodium did not change significantly during administration of the hormone, although some "rebound" was present on the day after the steroid was stopped. This urinary pattern was felt to be expression of age and had not been previously recorded in the literature as most or all patients studied in the past were adults.

PATIENTS WITH CYSTIC FIBROSIS: Further observations were undertaken in children with cystic fibrosis and the group was extended to include post-pubertal adolescents with this disease.

The adolescents with CFP had a renal response in all ways comparable to that of the control adults. This confirmed that the apparent lack of kidney response to aldosterone administration previously observed in children with CFP was related to the age of the patients tested and not to the underlying disease.

Other Investigations in Cystic Fibrosis

LATE INTESTINAL COMPLICATIONS OF CYSTIC FIBROSIS. The best known surgical complication of CFP is intestinal obstruction in the newborn period, so called, "meconium ileus". Regardless of age, however, all patients with CFP are subject to obstructive symptoms requiring careful surgical evaluation because of the abnormal composition of their stools.

Five such patients, 13 months through 31 years of age, have been seen at the NIH. Two responded to medical measures; three necessitated surgical intervention. Observation of such cases has afforded us an opportunity to understand the physio-pathologic mechanisms involved and given arise to important diagnostic and clinical considerations. Guiding principles for medical and surgical management have been outlined, based on our own experiences and a review of 23 cases from the literature. It is expected that this study will be of great diagnostic and therapeutic assistance to

physicians and surgeons throughout the country.

NEW SYNDROMES OF PANCREATIC DEFICIENCY SIMULATING CYSTIC FIBROSIS. It is now becoming apparent that patients thought to have cystic fibrosis on the basis of pancreatic insufficiency and chronic pulmonary disease, but with normal sweat electrolyte, may in reality represent one or more separate nosologic entities. Elevated sweat electrolytes may be a 100% requirement for the diagnosis of fibrocystic disease of the pancreas.

Two such patients have been extensively studied on our wards. One is a 24 year white male who has pancreatic deficiency, microcephaly, dwarfism, deafness, hypothyroidism, and chronic lung disease and 47 (XXY) chromosomes as in the Klinefelter's syndrome. Sweat sodium and chloride are normal. Extensive metabolic, endocrinologic and pathologic studies have been performed. This represents the first case of pancreatic insufficiency in the presence of a chromosomal anomaly.

The second patient is a 16 year old white female who has a history of pancreatic insufficiency and dwarfism, normal thyroid and pituitary function and a normal chromosomal pattern. In addition, she also has hypersplenism and bone marrow hypoplasia. A younger brother died a year ago of a syndrome consisting of pancreatic insufficiency, aplastic anemia, and dwarfism.

There is apparently a fairly large number of such patients throughout the country and further studies are planned in order to elucidate these interesting new syndromes.

PREGNANCY AND DELIVERY IN PATIENTS WITH CYSTIC FIBROSIS WITH A NATIONAL SURVEY. This study initiated last year has been continued. Two such patients have been observed at the NIH. The first patient, a 19 year old white female had an uncomplicated course and delivered a normal birth term male infant. The second patient, a 32 year Negro female aborted a stillborn male infant apparently free from cystic fibrosis on her 31st week. The mother expired four days post-partum and autopsy findings were confirmatory of the diagnosis.

A survey conducted throughout the United States has revealed seven additional cases, the

findings of which are being evaluated at the present time. It is expected that this study will give rise to important genetic and practical clinical considerations.

THE EFFECTS OF ANABOLIC STEROIDS ON THE COURSE AND PROGNOSIS OF PATIENTS WITH CYSTIC FIBROSIS. An evaluation of the effects of anabolic steroids was undertaken, their action in stimulating increases in body weight

are well known and their success in alleviating complications of pulmonary diseases have been reported.

Findings to date suggest that most of the CFP patients respond successfully to intermittent dosages of anabolic steroids with marked improvement of the nutritional state, but to a much lesser extent in the pulmonary involvement.

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

INTRODUCTION

With Dr. Dorland J. Davis' appointment as Director, NIAID, the intramural program of this Institute has been under the temporary direction of Dr. Leon Jacobs, who has served as Acting Scientific Director since October 1964. Because of the momentum of the research program, these changes have been effected very smoothly and the scope of NIAID activities has continued to expand. Characteristic of fundamental probing into different problems is the rapprochement of fields, which at the molecular level come closer together conceptually and technically. This is occurring in many areas of the NIAID program, such as in the biology of viruses, viral oncogenesis, bacterial metabolism, immunogenetics and immunochemistry, and even in the metabolism and structure of fungal and parasitic organisms. Considerable progress has been made in these areas, which have been, and will continue to be, encouraged in their development.

These fundamental aspects are only one part of the balanced NIAID programs. We also have supported epidemiological studies on viral, mycotic, and parasitic infections, the development of respiratory vaccines, clinical studies leading to accurate description of respiratory disease due to viruses and other agents, chemotherapy of systemic fungal disease, drug resistance in malaria, and a host of other problems too diverse to be summarized briefly here. They form important parts of our broad attack on infections and immunologic disease.

As our laboratory research produces findings that appear directly translatable into methods of disease control, we have made efforts to focus on definite goals with selected projects. The foremost example of this is the development of respiratory vaccines. Human volunteer studies for the assessment of the protective

value of vaccines have been performed under carefully controlled conditions at the Clinical Center and elsewhere, and have been followed by field trials in propitious epidemiological situations. The respiratory vaccine effort is an excellent example of what we consider the proper utilization of our Collaborative Program. We have excellent support for the developmental work on vaccines under contract, because of the interest of our intramural staff in the operation. The same sort of effective collaboration continues to be profitable in the Research Reference Reagents Program, and is being developed by the involvement of intramural personnel in the immunology and tissue transplantation program.

Another example is a project in Egypt, where a field trial is under way to evaluate mass treatment at subcurative, non-toxic, drug levels as a means of breaking transmission of schistosomiasis. This is being supported by PL 480 funds, and is carefully supervised by our intramural staff.

As has been pointed out previously, our direct research interests are so broad and challenging that we have characteristically sought opportunities to study infectious diseases at geographic locations where the agents can be observed under natural conditions. Our Middle America Research Unit in the Panama Canal Zone has produced very important results on the etiology, epidemiology, and control of Bolivian hemorrhagic fever and is concerned in general with arboviruses of importance in the tropical Americas. It has also been the site of highly productive epidemiological investigations on histoplasmosis. We are also supporting a small staff in Hawaii, the Pacific Research Section of LID, for investigations on the epidemiology of eosinophilic meningitis, which has been related to the aberrant wanderings of lar-

vae of a rat lungworm. In addition to the Egyptian project mentioned above, work is proceeding on an amebiasis study in India, which became functional this year, and on several other cooperative projects supported under the authority of PL 480. It should be emphasized that the NIAID scientists involved regard PL 480 projects as extensions of our intramural program and accept responsibility for the proper prosecution of the research. It is because of this attitude that we have attempted to maintain a balance in respect to the number of opportunities taken to extend or to undertake new lines of study under the aegis of PL 480 and the extent to which activities of this nature detract from ongoing programs at Bethesda.

A major change in our field stations was the closing of the Far East Research Project in Kuala Lumpur, Malaysia, in November 1964. This unit had been engaged in field studies especially to evaluate the relationship of simian to human malaria. Some of its operations have been transferred to our field station at Chamblee, Georgia.

As in the past, the Institute has continued its contributions to the training of biomedical scientists, not only by means of the Associate Program but by employment of Visiting Scientists and by playing host to numerous Guest Workers.

This year we are experiencing the loss of four senior investigators who are retiring to accept academic appointments. Dr. John P. Utz is leaving the Laboratory of Clinical Investigations for a professorship of medicine at the Medical College of Virginia. Dr. Leon Levin-tow leaves the Laboratory of Biology of Viruses for a professorship at the University of California, and Dr. Sheldon Dray is assuming the chairmanship of the Department of Microbiology at the University of Illinois College of Medicine, creating a vacancy in the Laboratory of Immunology. Dr. Arthur K. Saz left his position as Head, Medical and Physiological Bacteriology Section, LID, for the chairmanship of the Department of Microbiology and Tropical Medicine, Georgetown University School of Medicine. Each one of these men has become a recognized authority because of work done at Bethesda. We do not hope to recruit equally ac-

complished investigators; rather, we will sponsor the development of new personnel with the aid of our present cadre. Our experience in recruiting staff for the burgeoning fields of immunochemistry and molecular biology of viruses leads us to believe that our best chance to further our own work in these fields is by proper selection, training, and support of promising young individuals.

The following pages describe some of the principal aspects of the intramural research accomplishments.

LABORATORY OF PARASITE CHEMOTHERAPY

The major research effort to the staff continued to be centered on problems in malaria. Parasite resistance to antimalarial drugs in certain endemic areas continues to be a serious problem, and one which has dictated a renewed and continuing interest in chemotherapeutic investigations and other aspects of malaria. The expanded use of many of the simian malarias under experimental conditions has added greatly to the program of clinical evaluation of antimalarial agents in man and basic biological studies of the malarias of lower animals.

The single major change in the organization of the Laboratory during the year was the closing of the Far East Research Project in Kuala Lumpur, Malaysia, in November 1964. The staff of this Project was reassigned to the Chamblee, Georgia, Laboratory.

Malaria—Human

In the clinical testing of antimalarial drugs in human volunteers, emphasis has been placed on strains of parasites resistant to certain compounds and on possible methods for overcoming the resistance problem. Resistance to chloroguanide was induced in the Chesson strain of *Plasmodium vivax*. The resistant strain developed normally in volunteers treated with cycloguanil pamoate (CI-501), indicating that CI-501 would have no demonstrable effect on vivax strains which are resistant to the parent compound, chloroguanide. Studies have continued on a Southern Rhodesian strain of *P. falciparum*, known to be at least partially resistant to chloroguanide; CI-501 appears to be a causal

prophylactic against sporozoite-induced infections with this strain, but fails to protect against challenge by parasitized blood, indicating an incomplete resistance to the drug. The level of resistance has been increased through use of non-curative treatment with chlorguanide, and studies are under way to assess the effect of CI-501 on parasites of this strain with increased levels of resistance.

Field evaluation of CI-501 in Pakistan was terminated when resistant strains of *P. falciparum* were unexpectedly encountered. The suspected strain was isolated and established in volunteers and was subsequently found to be resistant to chlorguanide and pyrimethamine but susceptible to chloroquine, mepacrine and quinine.

Urinary excretion studies on CI-501 have confirmed the relationship of excretion rates to particle size of the drug. The duration of protection against malaria by this repository preparation is directly related to the excretion curves.

Because a number of strains of *P. falciparum* are now known to be susceptible only to quinine, and because the available supplies of quinine are limited, a study was undertaken to determine the minimal dose of quinine needed for suppression of sporozoite-induced infection. The currently recommended dose of 10 grains (648 mg) daily appears to be necessary for consistent suppression; 7.5 grains daily was inadequate.

In an effort to find additional antimalarial agents to be used alone or in combination with presently known compounds against resistant strains of malaria, investigations were done on sulfones and sulfonamides known to have antimalarial qualities. Diaminodiphenyl sulfone (DDS) was used against the Cambodian I strain of *P. falciparum*, known to be resistant to chlorguanide and pyrimethamine; to date (69 days after exposure) the medicated volunteers show no evidence of infection, indicating some promise of suppressive cure of some resistant falciparum strains. Sulfadiazine and sulfamethoxypyridazine, alone or in combination with pyrimethamine, were used against the Malayan III strain of *P. falciparum*, known to be resistant to all commonly used antimalar-

ials except quinine. Results have been encouraging in the treatment of this strain and the study is being extended.

Serial immuno-electrophoretic studies on the sera of 23 volunteers infected with *P. vivax* and 26 volunteers infected with *P. falciparum* have confirmed the correlation of the early change in serum proteins with an increase in the macroglobulin fraction. Reinfection studies, to determine the extent of acquired immunity, have been done with Chesson *P. vivax* and Southern Rhodesian *P. falciparum* and correlation of immunity with serological changes noted. In no case was the acquired immunity absolute, and in many the early attack of malaria was equal in intensity to that seen in non-immunes.

Malaria—Simian

Studies have been undertaken to determine if prior infection with human malarias might preclude subsequent susceptibility to the simian malarias in human volunteers. To date it appears that infection with *P. vivax* may prevent subsequent infection with the RO strain of *P. cynomolgi*, but in a single trial subsequent infection with the B strain of *P. cynomolgi* was successful. Continued efforts to infect human volunteers with *P. fieldi*, *P. inui*, *P. coatneyi* and *P. shortti* have to date been unsuccessful.

The natural vectors of simian and human malarias in the major ecological areas in Malaysia have been identified, and the natural prevalence rates of simian and human malarias determined. Isolation of parasites from simians in Southeast Asia has resulted in the description of several new species of *Plasmodium* and several additional species remain to be described. Simian malarias have been characterized on the basis of patterns of parasitemia, host cell preferences and pre-erythrocytic schizogony.

Rhesus monkeys infected with *P. cynomolgi* have shown an acute, self-limited thrombocytopenia, associated with high levels of parasitemia, and accompanied by leucopenia and inhibition of reticulocytosis. Study of the mechanism involved indicates multiple factors in pathogenesis. These findings have confirmed and

extended the limited observations in human malaria.

Entomology

Six to eight species of *Anopheles* have been maintained and utilized to determine their comparative susceptibility to the simian and human malarias. Transmission of *P. brasiliense*, *P. cynomolgi* (several strains), and *P. shortti* through several anophelines has been obtained. Several strains of *P. inui* from Southeast Asian areas have been found infective to *A. maculatus*, *A. stephensi* and *A. freeborni* and transmission through these species has occurred. Attempts to infect mosquitoes with *P. coatneyi*, *P. fieldi* and *P. knowlesi* have been successful, but quantitatively disappointing, and attempts to transmit the infections have failed thus far.

Immunology

Studies of fluorescent antibody response with ten species of monkey malaria, four species of human malaria, and one species of bird malaria indicate significant cross-reactivity. Antigen derived from *P. fieldi* infections in monkeys cross-reacts highly with most of the other species and appears to be useful for routine determinations of immune status of hosts previously infected with other species. *P. brasiliense*, a quartan malaria of South American monkeys, provides a significant cross-reaction with the quartan species of man, *P. malariae*. With *P. gallinaceum*, it has been possible to stain all parasite stages in the chick and in the mosquito using a single homologous direct fluorescein-conjugated gamma globulin. Staining in all stages was cytoplasmic, but not nuclear.

In monkeys infected with *P. cynomolgi* there is a significant delay in the appearance of antibody in splenectomized animals.

Using disc electrophoresis on acrylamide gel and extracts of *P. berghei* and *P. vinkei* parasite suspensions, 15 and 7 protein fractions, respectively, could be identified as being of parasite origin.

Chemotherapy

Of 158 new compounds tested against *P. berghei* in mice, 17 sulfone derivatives were

variably effective. One sulfone has been normally effective against a chloroquine-resistant strain, but did not appear to potentiate chloroquine when used in combination with this drug against the same strain. Silicon rubber capsules containing pyrimethamine implanted in chickens, mice, and monkeys protected these hosts from their respective malarias; apparently enough of the drug is passed through the capsule wall to provide adequate plasma levels for relatively complete suppression of the infection.

Strains of *P. berghei* have been developed which are totally resistant to chloroquine, pyrimethamine and quinine, i.e., parasites develop and persist normally in the face of drug dosages which are lethal to 50% of the host animals. This is the first time that a relatively stable chloroquine-resistant strain of any malaria has been produced experimentally. Both the quinine- and chloroquine-resistant strains are characterized by the virtual absence of malarial pigment, and both strains are considerably less lethal to mice than the parent strain. The pyrimethamine-resistant strain shows no loss of virulence and the pigment-production is normal.

Biochemistry

Reversal of the antimalarial activity of chloroquine or pyrimethamine occurs with the addition of ferrihemic acid (hematin). When the two antimalarials are used in combination, this reversal is not present to a significant degree. Chloroquine has been found to reduce oxygen uptake, and aldolase activity of *P. berghei*. With various fractions of parasite suspensions, chloroquine increases the uptake of phosphate in the acid soluble fraction and reduces phosphate uptake in the RNA fraction; uptake into the phospholipid and DNA fractions was not significantly altered.

A technique for detection of low levels of chloroquine in plasma, using gas-liquid chromatography, has been devised which compares favorably with the spectrofluorophotometric method, at present the only one available. The new method has been used only with control solutions and further studies will be required to extend its use to tissue fluids.

LABORATORY OF TROPICAL VIROLOGY

Serology

Hemagglutination and hemagglutination-inhibition for Tacaribe, Junin and Machupo viruses is a significant advance. Although the epidemiological usefulness of HI remains to be demonstrated, present evidence is comparable to previously recognized CF relationships.

Fortunately, chloroform-treated Machupo antigens were found suitable for both CF and HA tests. Treatment with chloroform has the additional advantage of reducing infectivity, but incomplete inactivation was not obtained. Ethylene oxide-inactivated virus suspensions were equally useful as CF or HA antigens. Their infectivity (or safety relative to CHCl_3 antigens) remains to be evaluated. The relative infectivity of antigen treated by various means is of considerable importance because the distribution of infectious Machupo virus materials is limited by inherent hazards to health. Availability of demonstrably safe antigens for general distribution would be advantageous.

Tissue Culture

A suitable TC system for TC CPD_{50} assays of Machupo virus infection has not been demonstrated, even after screening more than 35 different cell culture types. Plaque-formation has been demonstrated in several TC systems but techniques are variable, cumbersome and expensive. However, in the absence of reproducible CPE, plaque assays still represent our most useful tool.

Demonstration and recovery of PPLO from most WI-26 cultures obtained locally provided a most likely and interesting explanation for interference with assays of CPE in WI-26 cultures.

Immunity

Although immunity to challenge by Tacaribe virus was previously demonstrated by transfer of maternal antibody to four-day-old suckling hamsters, similar experiments with Machupo virus have been unsuccessful. Persistent chronic infection and/or unsuitable laboratory hosts are the most likely factors responsible for un-

satisfactory results. It becomes increasingly apparent that challenge experiments in primates are indicated.

LABORATORY OF BACTERIAL DISEASES

Mycoplasma

The basic studies on *Mycoplasma* (Pleuropneumonia-like Organisms) have progressed satisfactorily during the past year. New information has accrued on the pathogenicity of certain animal and human species, and the interrelations between many members of the myco-plasma group have been clarified.

A comparative study of the characteristics of 17 recognized strains of mycoplasma initially recovered from rats and mice was completed. Five major serological types were recognized. Of particular interest was the finding that the culture isolated by Nelson, from an outbreak of conjunctivitis in mice, types as *M. neurolyticum* and produces the neurotoxin characteristic of this species. Strains of *M. pulmonis* from the lungs of rats and mice were indistinguishable. *M. arthritidis* (rat) was found to be serologically similar to strains designated as *M. hominis* type 2.

Mycoplasma gallisepticum has been shown to produce lesions in germfree chickens similar to those observed in conventional chickens, thus establishing the basic pathogenicity of this organism. Although specific lesions were noted, together with recovery of the organism, full-blown overt clinical disease did not develop. Whether the presence of other organisms in conventional chickens contributes to the development of chronic respiratory disease (CRD) is still an open question. In addition to the production of specific lesions, spread to the uninoculated cage mates and long-term (22 weeks) persistence of infection were established.

The behavior of *M. pneumoniae* was studied in germfree guinea pigs. The organism was regularly isolated from all test animals during a four-week period and minimal pneumonic lesions were produced. Spread of the infection to uninoculated animals was slight.

The extracellular neurotoxin produced by *M. neurolyticum* has been further studied. Lysis of cells by a variety of methods results in de-

struction of the toxin. Partial purification has been obtained by Sephadex fractionation. The activity is contained in a high molecular weight (>200,000) fraction consisting primarily of protein.

M. neurolyticum has been adapted to growth in several types of tissue culture cells. In the first attempt a non-toxicogenic strain resulted which grew in serial passage but did not produce a cytopathogenic effect on the cells. Subsequently, a cell-adapted strain which produces toxin and CPE has been carried through 44 serial passages in tissue culture with a gradual increase in neurotoxicity for mice. Although no growth occurred in tissue culture medium without cells, extracellular mycoplasma were numerous in the tissue cultures, and upon repeated examination intracellular forms could not be demonstrated.

A *Mycoplasma* strain was isolated from a febrile patient who had recently undergone gynecologic surgery. The organism was identified as *M. hominis* type 1. There was an accompanying antibody rise in the patient's serum. The isolation of this organism from the blood stream, in association with clinical illness, provides further evidence of the pathogenicity of *M. hominis* type 1, for man.

In collaboration with a scientist in LPD, a strain of *Plasmodium berghei* which, after passage through mice, acquired the ability to produce paralysis in rats, was examined for mycoplasma. Toxin-producing *M. neurolyticum* was recovered from passage material used to induce *Plasmodium berghei* infections in rats. The mycoplasma is probably type A and produces toxin typical of the species. When this organism was mixed with a clean strain of *P. berghei*, and inoculated into rats the paralytic syndrome was reproduced. *Mycoplasma* has not yet been recovered from paralyzed rats. As rats are considerably more resistant to *M. neurolyticum* neurotoxin than mice, it appears that the combination of malaria and mycoplasma was necessary for the appearance of the paralytic syndrome.

Brucella

In collaboration with a scientist of LBV, a study of DNA homology has been used to

define the genus *Brucella*. The results reveal that there is a high degree of homology of DNA within the genus. This includes *Br. neotomae*. *Pasteurella tularensis* gave no specific binding. The organism of ram epididymitis is related to, but not homologous with *Brucella*.

LABORATORY OF GERMFREE ANIMAL RESEARCH

Autoallergic Disease Produced in Germfree Animals by Germfree Tissue Extracts

Germfree guinea pigs were inoculated with extracts, plus adjuvant, of thyroid, testes, or spinal cord, prepared from other germfree guinea pigs. In due course, each group of animals developed autoallergic disease, i.e., either thyroiditis, aspermatogenesis or encephalomyelitis. Control animals injected with lens material did not develop disease. Attempts to isolate PPLO, L forms and bacteria from the extracts and target organs of the immunized animals failed.

Immunoglobulins in germfree mice

Immunoglobulins can be characterized and quantitatively measured by use of specific antisera. In addition, sites of synthesis can be determined by incubating tissues in a medium containing C-14-labeled amino acids, and subsequently analyzing culture fluids for labeled proteins by means of immunoelectrophoresis and autoradiography. It has been shown that germfree mice usually synthesize and contain in their sera only 7S- γ_1 and γ M immunoglobulins in about 10% of the amounts found in conventionally reared mice. 7S- γ_2 or γ A immunoglobulins are not present, but their synthesis can be readily stimulated by the administration of antigen or bacterial lipopolysaccharide. After such stimulation, serum immunoglobulin levels are very high when compared to controls. These studies afford the possibility of investigating immune responses with an extremely low "background" level of immunoglobulin synthesis in control animals.

Eaton Agent Transmitted to Germfree Guinea Pig

In a study designed to determine the pathogenicity of Eaton Agent, *Mycoplasma pneumoniae*, in young germfree and conventional guinea pigs, a heavy inoculum of *M. pneumoniae* was administered intranasally to each group. Uninoculated animals in each cage served as contact controls. Samples of nasal exudate were taken one day post-inoculation and then weekly for a period of four weeks. *M. pneumoniae* was recovered from all inoculated germfree guinea pigs at every nasal sampling, whereas only a few positive cultures were isolated from the conventional animals. Contact transmission of Eaton Agent appeared very limited. Gross lesions in the infected animal consisted of a patchy interstitial bronchial pneumonia. The germfree guinea pig thus provides another experimental host for the study of the etiologic agent of primary atypical pneumonia.

New Immunoglobulin Detected and Characterized in Immunized but not Germfree Mice

A new immunoglobulin sub-class, not previously identified, has been found in the sera of mice immunized with a variety of pure antigens. It is not found in normal or germfree mice. The protein, designated " γ_3 ", was identified by immunoelectrophoresis using appropriate antisera. It was possible to obtain a specific antiserum by absorption with myeloma proteins. The new protein appears to cross-react with 7S- γ_2 immunoglobulin, binds specific antigen in immune sera, migrates with the very slow 7S- γ_2 globulins, and has an estimated size in the range of 7S globulins.

Antibody Response and Serum Protein Changes Determined in Monocontaminated Germ-free Mice

The delayed antibody response of germfree mice has been followed by sensitive bactericidal assay. Germfree mice fed viable *Escherichia coli* and another germfree group given a single subcutaneous injection of the same strain of heat-killed *E. coli*, showed peak antibody titers within two weeks. This peak titer

conventional controls reached similar levels of was maintained over a four-week experimental period. In contrast, subcutaneously injected bactericidal antibody in four days, eventually dropping to one-fourth of this level by the fourth day. Immunoelectrophoretic analyses of serum revealed a new band and determined time relations in the appearance of various globulins.

High Macroglobuli Levels Found in Human Malaria

Immunoelectrophoretic patterns have shown an accentuation of the IgM or 19S-globulin lines in individuals infected with malaria. Quantitation of this immunoglobulin was determined by a gel diffusion technique using serum from volunteers with sporozoite- or blood-induced vivax malaria. The correlation between increase in malarial antibody and increase in IgM macroglobulin was quite striking. In a relapse case, IgM macroglobulin production in the second attack appeared to be the same as in the first attack.

Adoptive Transfer of Autoallergic Thyroiditis by Means of Lymph Node Cells

To elucidate the mechanism of allergic tissue destruction of grafts, tumors, and the bodies' own organs, attempts have been made to devise a model whereby the self destructive disease, autoallergic thyroiditis, can be transferred by means of lymphoid cells from immunized to otherwise untreated recipients. Strain 13 histocompatible guinea pigs were immunized with thyroid antigen and cells from their lymph nodes were transferred to other Strain 13 animals. Since the cells were in a histocompatible host, they could survive and function. The incidence of disease in all these recipients was 27%. By using donors which were both immunized and later skin-tested, a higher incidence of disease was obtained; the incidence of thyroiditis in the recipient animals was 66%. A 4% incidence of disease was produced in the control recipients, outbred Hartley guinea pigs, which kill the injected cells and serve as a control for any antigen which might accompany these cells. This is the same as the background incidence of disease in animals given large

doses of leukocytes derived from animals immunized with other non-tissue antigens. With the improvement of this transfer model, it will be possible to analyze the effects of different types of immune factors on tissue destruction.

Allergic Encephalomyelitis Produced in Newborn Monkeys

The finding that a chronic form of experimental allergic encephalomyelitis could be produced regularly in neonatal Strain 13 guinea pigs provided an experimental model more closely resembling human demyelinating diseases (multiple sclerosis, Schilder's disease, etc.) than had previously been available. This prompted the institution of an intensive study of allergic encephalomyelitis in monkeys in collaboration with members of the Laboratory of Perinatal Physiology, NINDB, in San Juan, Puerto Rico. Six newborn monkeys immunized with spinal cord antigen and Freund's adjuvant all developed clinical neurological signs and symptoms, some of which were quite delayed. Several of the animals have shown clinical remissions and exacerbations of their disease similar to multiple sclerosis. Four of the six animals died, after exhibiting severe clinical, neurological disorders with extensive pathologic involvement of the CNS, including the optic nerve, retina, and uveal tract. This work provides an extremely valuable experimental model for the study of multiple sclerosis and other human demyelinating diseases, and of human ocular disorders of possible autoimmune etiology.

Genetic Basis of Allergic Encephalomyelitis Delinieated in Guinea Pigs

Utilizing inbred and randombred guinea pigs a genetically controlled difference in the production of experimental allergic encephalomyelitis has been demonstrated. Strain 13 animals are susceptible and Strain 2 animals are resistant to this disease. F_1 animals derived from crosses between the two strains were immunized with spinal cord and Freund's adjuvant, and 100% of the animals developed allergic encephalomyelitis. This demonstrates the dominant phenotype to be susceptibility, rather than resistance, to allergic encephalomyelitis.

The information on the factors affecting the production of experimental allergic encephalomyelitis, as derived from the study of such backcrosses, should prove valuable in understanding the mechanisms operating in autoimmune disease in general.

Thyroid Transplantation Studies in Inbred Guinea Pigs

In order to assess certain factors influencing transplantation immunity, whole thyroid glands were employed in transplantation experiments. Transplants in Strain 13 inbred guinea pigs were compared to those in Hartley random-bred guinea pigs. Deep intermuscular sites were found to be the most suitable for organ transplantation. The inbred Strain 13 guinea pigs have not shown complete organ compatibility, although they have been reported to be skin histocompatible. After six weeks, occasional Strain 13 isografts have shown mononuclear cell infiltrates. This infiltrate was qualitatively similar to that found in Hartley strain homografts in which some of the grafts had survived for six weeks. In no case so far studied, has grafting of normal thyroid tissue produced any histologic changes in the thyroid tissue of the host.

LABORATORY OF IMMUNOLOGY

Induction of Homograft Tolerance

Immunization of donor mice with any one of a variety of antigens has been found to modify considerably the immunologic competence of their lymphoid cells transferred to sub-lethally x-irradiated allogenic recipients. Diminution or total suppression of the otherwise uniformly fatal graft versus host reaction was achieved. A partial state of transplantation tolerance was generally produced as evidenced by the subsequent acceptance by irradiated animals of skin grafts from such immunized donor mice. Heretofore, most efforts in transplantation research have been directed to treatment of the host (recipient) in whom it was sought to induce some degree of tolerance to donor tissues. This work, in focusing attention on effecting significant changes in the immunologic capabil-

ties of donor lymphoid cells at the time of this transfer, differs conceptually from previous efforts in this field and offers new possibilities for coping with the immunologic rejection mechanism.

Antibody-Induced Unresponsiveness

Specific antibody passively administered prior to primary antigenic stimulation was shown to result in immunologic suppression. The quantity of specific antiserum which sufficed to suppress the appearance of antibody-forming cells was remarkably small. These findings may be of particular relevance to analyzing neonatal tolerance inasmuch as neonates generally maintain significant levels of maternal antibodies for appreciable periods after birth.

Genetic Basis of Hypersensitivity

Strains of highly inbred and histocompatible guinea pigs were found to differ strikingly in their susceptibility and resistance to hypersensitivity reactions. At least one major basis for this difference in reactivity could be the amount of lung histamine available for release. Hartley strain guinea pigs were found to have up to three times as much histamine in their lungs as Strain 2 animals. Inasmuch as the amount of non-liberated histamine remained constant during anaphylactic shock, Hartley pigs could liberate up to ten times as much histamine as Strain 2 animals. The amounts of liberable histamine were found to be correlated with the number of mast cells present in lung tissue.

Depending on the amino acid composition and molecular weight of various synthetic amino acid polymer antigens, major differences were found between Strains 2 and 13 guinea pigs in their capacity to respond, as measured by skin sensitivity and circulating antibody. Strain 2 animals reacted fully to the glutamine-leucine-alanine polymers as contrasted to a total lack of responsiveness of Strain 13 animals. It remains for work in progress on first and second generation hybrids to establish this definitively as a genetic basis for the immune response.

RNA Role in Immune Response

The role of ribonucleic acid fractions in immune reactions is under study, utilizing ϕ X174 bacteriophage as antigen and the highly sensitive and objective phage-neutralizing assay for antibody. RNA extracted from macrophages that had been briefly incubated *in vitro* with phage was capable of inducing a primary immune response by normal guinea pig lymph node cells in peritoneally implanted millipore chambers. The characteristics of this situation are under study in parallel with the effects of RNA extracted from lymphoid tissues of immune animals, added to normal spleen cells and maintained in tissue culture. Further investigation of these two kinds of test situations may eventually lead to a decision as to whether the process of antibody synthesis involves induction by RNA-antigen complexes or transformation of normal lymphoid cells by immune RNA.

Characteristics of Unique Response to Enterobacterial Antigen

Antibody-forming cells were discernible as early as 14 hours after a systemic injection of microgram amounts of *Salmonella* polysaccharide. Circulating antibody was first detectable at 60 hours; peak response was at 5 days. Significant levels of circulating antibody still persisted a year after a single minute antigenic stimulus; a recall injection led to a greatly augmented immune response. In both instances the type of antibody present was *exclusively* of the 19S variety. During early phases of the primary and secondary response 5–10% of the total number of antibody-forming cells were found in peripheral blood. The thymus was also involved, as evidenced by loss of cellularity accompanied by a transitory infiltration of antibody-forming cells and subsequent restoration of thymic cellularity.

Time Factors in Immunosuppression by Drugs

Both the primary and secondary phases of the antibody response could be suppressed by methotrexate provided administration of the drug was begun at or near the time of antigen administration. Indeed, even administration later on in the latent or inductive phase or in

the early log phase of antibody production resulted in marked inhibition. The time for suppressing the secondary response was more critical, but even here treatment within two days of administration of antigen effectively suppressed antibody production. Once a steady state of antibody production was achieved, methotrexate, even in high dosage, was without significant effect on antibody levels. Delayed skin reactivity to bovine gamma globulin and to tuberculin could be suppressed by methotrexate, but not as effectively as antibody production. Perhaps the most impressive findings were those in which specific immune unresponsiveness (tolerance) could be induced by prolonged administration of methotrexate after a single large dose of antigen. The studies are important for clinical use of antimetabolites in the treatment of autoimmune states and for the suppression of the homograft reaction.

Characterization of Active Moiety in Skin-Sensitizing Antibodies

The skin-sensitizing antibodies in the serum of individuals with atopic allergies have been further investigated to determine whether their very considerable carbohydrate moiety is involved in hypersensitivity reactions. Sera of ragweed-sensitive patients were exposed to neuraminidase and to periodate. Enzymatic cleavage of neuraminic acid did not alter antibody reactivity but periodate oxidation leading to marked reduction in fucose, sialic acid, hexose and hexosamine resulted in reduction of skin-sensitizing activity.

Maternal Antibody Affects Gene Expression

Maternal anti-allotype antibody specifically inhibits the genetically defined synthesis of the corresponding allotype of the fetus. As markers the γ -globulin allotypes permit differentiation of passively transferred maternal γ -globulin from newly synthesized γ -globulin in the serum of the neonatal rabbit. It was found that rabbits of genotype b4-b5 received maternal anti-5 during fetal life from b4-b4 mothers immunized with b5 immunoglobulin. During a period which has now reached three years, it was found that a substantially lower percent of the total γ -G in the b4-b5 offspring has the b5

allotype, compared with the b4-b5 offspring of non-immunized b4-b5 mothers. The same effect was obtained when b4-b5 offspring of non-immunized mothers were injected with anti-b5 antiserum shortly after birth. In these offspring the total γ -G was normal; a compensatory increased production of the alternative allelic allotype accounted for most of the γ -G in their sera. There thus exists an important mechanism for controlling the proportion of given allotypes making up the total immunoglobulin.

Amino Acid Sequences of Gamma Globulin

The nature of the chemical differences associated with different allotypic specificities of rabbit γ -globulin have been subjected to intensive study. Heretofore, the allotypes of light chains from γ -G immunoglobulin could be recognized by specific antisera. By analysis of the light chains obtained from several b4 and b5 homozygous rabbits, significant differences were consistently found in the amino acid composition of these light chains controlled by different allelic genes. The differences amounted to 15 residues per light chain and involved at least 8 amino acids. No significant differences were found for the remaining amino acids. The observed differences are considered to indicate at least some of these amino acids may be a part of the structural configuration of the antigenic determinants.

LABORATORY OF CLINICAL INVESTIGATIONS

Adenovirus Groups and Vaccines

As a result of volunteer studies it was found that human adenovirus serotypes may be divided into three immunologic groups which correspond with Rosen's earlier rat and rhesus monkey erythrocyte agglutination groups. One of those, group 3, contains adenovirus serotypes 1, 2, 4, 5 and 6.

In a separate study a purified soluble protein antigen from type 1 adenovirus was shown to be protective against challenge with this agent. This antigen produced a high frequency of antibody responses in human volunteers to serotypes in group 3 (1, 2, 4, 5 and 6). Review of

the literature suggests that heterotypically induced antibody in this group is protective against illness and infection. The clear possibility thus exists that a vaccine prepared from adenovirus type 1 may induce protection against types 1, 2, 4, 5 and 6. Such group-wide protection would offer an extremely efficient and safe means of vaccination.

Equine Influenza in Man

Five antibody-free volunteers inoculated with equine influenza virus became infected, and one of them became ill with acute influenza syndrome. Equine virus recovered from man was serially passed in Assateague ponies, with illness developing in 3 of 8, and infection without illness in 4 of the 5. At present it appears that equine virus has significant infectiousness for both man and horses.

Aerosol Transmission of Respiratory Viral Disease

Studies with the staff of the U. S. Biological Laboratories at Ft. Detrick have achieved very significant advances this year: 1) Studies of sneezes and coughs have shown that large numbers of particles in the range of 1-10 microns in diameter are discharged (± 1 million with a sneeze and $\pm 100,000$ with a cough). Assays for virus have shown from 50 to more than 1,000 TCID₅₀ of virus per sneeze. 2) Decay studies have shown significant retention of viability for two hours and longer in air similar in humidity and temperature to that in hospital rooms. Using a large volume electrostatic air sampler (10,000 liters per minute) large amounts of Coxsackie A-21 virus were recovered from hospital room air occupied by volunteers ill with this infection. 3) Of this agent, six TCID₅₀ could initiate infection and illness in a volunteer.

The dose to initiate infection with adenovirus is similar. With rhinovirus, it was found that the human infectious dose was 30-fold smaller than the least detectable amount of virus by conventional tissue culture techniques (human infectious dose ID₅₀ = .03 TCID₅₀). This in itself is a most fascinating finding and is, no doubt, one of the reasons why illness with this large group of viruses is so prevalent.

Non-Antibody Resistance to Rhinovirus Common Cold

Earlier studies in this laboratory showed that illness with a particular rhinovirus type was followed by nearly complete resistance to reinfection with the same strain for several weeks. This year studies were completed which revealed that this resistance occurs for illness with another rhinovirus type, but not for Coxsackie A-21.

Prior studies had excluded serum antibody as a basis for this resistance (as it is now measured in neutralizing antibody tests). Such a role has now also been excluded for nasal secretion antibody and probably for interferon. An elucidation of this factor of resistance will add a new facet of understanding to the problem of resistance to respiratory viral infection.

Gamma-G Immunoglobulin Structure and Antibody Synthesis

In immunogenetic studies in mice, five antigenic sites have been identified on the heavy chains of the 7S γ -G immunoglobulin molecules. Antisera to specific molecular types of myeloma immunoglobulins have further differentiated the 7S γ -G immunoglobulins into 2 distinct proteins, each containing a specific antigenic site for γ -G globulin synthesis. Close linkage in their genetic control has been shown.

Transplantation of Specific Genes Controlling γ -G Immunoglobulin Synthesis in Mice

Permanent survival of multiple skin grafts has been accomplished in inbred strains of mice that received lethal irradiation doses and then hematopoietic tissue pooled from a variety of mice containing different genes controlling γ -G immunoglobulin synthesis.

Antigenicity of Small Molecular Weight Drugs

It has been postulated that conjugation of sulfonamides to protein takes place via a reactive metabolite, namely, the N-hydroxyl derivative. A conjugate with thiolated bovine serum albumin has been made with such a derivative of sulfadiazine. With the conjugated protein as the test antigen it was demonstrated by passive

cutaneous anaphylaxis (PCA) and by bentonite agglutination test that specific antibodies were produced in rabbits sensitized directly with sulfadiazine. Inhibition of the PCA reaction and of the bentonite test by a univalent hapten prepared from a mercaptoamino acid and the reactive intermediate strongly indicated that a mercaptoamino acid and the reactive component part of the determinant group of the antigen formed *in vivo*. A similar reactive intermediate is considered to be the active carcinogenic form of certain chemical carcinogens.

Endotoxin Tolerance

Following a series of endotoxin injections, a state of resistance (tolerance) is induced to many of the biologic effects of these pyrogenic agents. Utilizing quantitative techniques, characterized in this laboratory, it has been shown that tolerance in rabbits persists for much greater periods of time than previously considered (at least 60 days). This refractory state can be passively transferred with serum; thus, a humoral factor is present. Extensive investigations here indicate that endotoxin antibody plays only a minor, if any, role in this tolerant state.

Antigen-Antibody Induced Fever

Rabbits immunized with human serum albumin (HSA) will respond to challenge with HSA by developing a fever. This pyrogenic reactivity is correlated with development of circulating antibody to HSA. Preliminary studies suggest that the antibody responsible for the fever is of the 7S gamma globulin type.

Recurrent Fever of Unknown Etiology

Extensive investigations of a large number of humans with "periodic fever" have revealed that these patients represent a diverse array of clinical disorders. Techniques have been developed to measure, with precision and sensitivity, plasma unconjugated etiocholanolone, a known pyrogenic steroid. At present it appears that this steroid may be non-specifically elevated in certain illnesses characterized by fever.

Treatment of Systemic Fungal Disease with Hamycin

In experimental infections in laboratory animals performed jointly with the section on Medical Mycology, LID, the new antifungal drug, Hamycin, was found more effective than any previously tested against *B. dermatitidis*, the causative agent of the severe pulmonary and cutaneous disease, blastomycosis, in man; and against *Cryptococcus neoformans*, the causative agent of the most frequently encountered fungal meningitis in man.

In studies at the Clinical Center, Hamycin was chemotherapeutically active in three of four patients with blastomycosis.

LABORATORY OF PARASITIC DISEASES

This Laboratory has been under the direction of Dr. Paul P. Weinstein, who is serving as Acting Chief during Dr. Jacobs' assignment as Acting Scientific Director. Despite some personnel changes, the program has remained essentially the same as in past years, except for certain shifts in emphasis due to particularly interesting and encouraging findings.

The significant findings of the past year are summarized below:

Vitamin B₁₂—Nematode Relations

The concentration of vitamin B₁₂ (cobamide) in the coelomocytes of nematodes is directly related to the synthetic capacities of the microbial flora upon which the larvae feed. *Nipponstrongylus* larvae grown on washed saline suspensions of single bacterial species capable of synthesizing cobamide in high concentration (*Propionibacterium shermanii*, *P. freudenreichii*, *Clostridium sticklandii*) show by visual inspection intense accumulation of cobamide in the coelomocytes. However, no such concentration occurs when larvae are fed *Escherichia coli* (wild type or auxotrophic mutants) incapable of synthesizing cobamide. Infective larvae maintained as quiescent "dauer" forms show no diminution of cobamide for several months. However, the pigment soon disappears from the coelomocytes of these larvae after they have begun *in vivo* development in the rat, or *in vitro* growth at 37°, suggesting utilization.

tion within the cell or dispersal of cobamide to differentiating tissue.

The conversion of cobamide to the metabolically active coenzyme form was shown to occur in pig *Ascaris* maintained *in vitro*, and further investigations will now be made to obtain information on the biochemical requirements for the large quantities of cobamide found in many parasitic helminths.

Thiabendazole, a broad spectrum anthelmintic, was found to inhibit the growth of *Ochromonas malhamensis*, a completely B_{12} dependent organism, and further study on the mechanism of inhibition is being conducted.

Schistosomiasis

1) *Pathology.* Little information on the relation of parasite burden to pathology is available for the human disease. The perfusion of cadavers in Brazil to recover *S. mansoni* adults has demonstrated heavier worm burdens (170-330 worm pairs) in cases of pipestem fibrosis than in cases without lesions (70 worm pairs maximum, 5-20 pairs most common). In one young individual with "subacute" schistosomiasis, several thousand worms have been recovered. Should the relative constancy of worm burdens obtained in patients with hepato-splenic schistosomiasis continue as a finding, then it would suggest a delicate balance of host-parasite interaction not previously suspected. Fecal and hepatic egg counts have correlated poorly, thus far, with the worm burdens obtained at autopsy.

2) *Clinical Field Studies.* A collaborative program being carried out in Puerto Rico has demonstrated that therapy based upon the slow intravenous infusion of the antimonial stibophen in only $\frac{1}{4}$ of the usual dose has a marked therapeutic effect, and that this regimen is well tolerated. An adjunct study being conducted in Egypt under PL 480 funds is attempting to evaluate the suppressive, therapeutic and prophylactic effect of Astiban (an antimonial) administered at monthly intervals to a population of over 5,000 individuals in an endemic area. Evaluation at the end of the first year of suppressive treatment indicates that less than 5% of the treated individuals are currently passing viable eggs of either *S. mansoni* or *S. haemato-*

bium, whereas prior to therapy more than 50% were positive. This type of treatment may prove to be an effective measure to interrupt transmission of the disease.

3) *Laboratory Chemotherapeutic Studies.* In experimentally infected mice, isotopically labeled antimony reached maximum concentrations in schistosome worms within 30 minutes following injection, and then fell to less than 50% of peak concentration within 8 hours. Such information allows for a more rational development of therapeutic regimens.

Angiostrongyliasis

The development of partial immunity to infection with the nematode *Angiostrongylus cantonensis*, implicated in the causation of eosinophilic meningoencephalitis in man in the Pacific, has been demonstrated in rabbits. After a primary infection, animals given a challenge infection developed only transitory CNS symptoms, but not paraplegia. The majority of previously unexposed rabbits, however, when given the challenge dose developed paraplegia.

Adult *Angiostrongylus cantonensis* was determined to have a pronounced rate of glucose absorption. Most of the glucose being metabolized aerobically or anaerobically was transformed into lactic acid. Some of the glucose was also synthesized to glycogen in the presence of CO_2 .

In studies on *Angiostrongylus cantonensis* infection in laboratory animals, antigen satisfactory for the hemagglutination test was obtained only from culture incubates of female worms; tests with such antigens were specific. Incubates of larval or male worms were not antigenic in this test. The positive serology is definitely correlated with patency of the infection in rats. Positive HA tests are not found, with incubate antigens, in animals that do not support the infection to patency. Whole worm antigens can reveal non-patent infections; however, they cross-react with other helminths.

Filariasis

Invasion of the CNS by nematodes has become increasingly recognized as a cause of disease in man and animals. *Dipetalonema vitei*

has been found to induce extensive central nervous system disturbances in the mongolian gerbil. These were produced by the invasion of the CNS by immature fifth stage or mature gravid females.

Suckling rats have been found to be suitable experimental hosts in which to study the early development of *Dirofilaria immitis*, the dog heartworm.

Effect of Host Diet on Parasites

In further studies on the mechanism of action of certain purified enriched low-residue diets causing rapid loss of both tapeworm and pinworm infections in mice, it was determined that: 1) The type of carbohydrate and fat incorporated in the diet does not affect the loss of infection. 2) Removing the vitamin pyridoxine from the purified diet and supplementing with the antimetabolite desoxyribozyme partially reverses the diets' effects. 3) The effects of the purified diets on 2 species of pinworms commonly found in mice were more marked in cases of *Syphacia obvelata* than with *Aspicularis tetraptera*.

Amebiasis

An important advance has been made in the axenic cultivation of *Entamoeba histolyticus*. A clear liquid medium has been developed which can be used for initiation of cultures and maintenance. Amoebae in culture can be counted by automatic, electronic equipment.

Analysis of the complex of large amoebae known as *E. histolytica* has distinguished two groups, one with growth restricted to approximately human body temperature and osmotic pressure, and another with the same capabilities, but able additionally to carry on all activities at 25° and in extremely hypotonic solutions. Infectivity studies in young rats indicate that members of the former group can colonize and invade the cecum, but not members of the latter. This implies that much previous work in epidemiology, chemotherapeutic studies, etc., may have to be reassessed.

Studies which were initiated in October 1964 in India (PL 480 project) were designed to obtain information concerning the *Entamoeba* complex, particularly in the area of the rela-

tionship of infection to disease, and to improve present capabilities in diagnosis, especially of amebic hepatitis and liver abscess. Personnel have been trained, working protocols have been established, and the preliminary results indicate a high prevalence rate of *E. histolytica* and *E. hartmanni*. Serologic study by fluorescent antibody and hemagglutination tests are now under way.

Investigation of fluorescent antibody procedures for identification of antibodies against *E. histolytica* has demonstrated that an avirulent strain of *E. histolytica* was utterly unsuitable as antigen in the FA serology of amebiasis, whereas antigen from a virulent strain worked quite well. Thus, although the FA method itself is at present not useful for diagnosis, it provides a rather simple means for testing the suitability of different ameba strains as antigens, for example, in hemagglutination tests.

Physiologic and Biochemical Studies

Larval and adult stages of the tapeworm *Taenia taeniaformis* were found to absorb more glycerol than glucose. The culture form of *Trypanosoma rhodesiense* withdraws glycerol almost exclusively from a mixture of glycerol and glucose, while *T. cruzi* does the reverse. These results re-emphasize the great metabolic differences already established between Group A and Group B trypanosomes. Appreciable sodium concentrations are necessary for maximal glucose absorption.

Continued investigation of the metabolic role of calcareous corpuscles in tapeworms using P^{32} has led to the conclusion that one function of these interesting structures is to act as a phosphate reserve on which the organism can draw for metabolic phosphate requirements. As a very important corollary, investigations on ion accumulation by isolated rat liver mitochondria have shown that the massive uptake of calcium and phosphate ions is accompanied by the formation of electron-opaque granules within the mitochondrion. By means of techniques used for the isolation of calcareous corpuscles from tapeworms, large quantities of dense granules have been obtained from mitochondria. These granules are composed of inorganic constituents (Ca^{++} , P_i , Mg^{++} , CO_3^{--})

and substantial quantities of organic materials. Studies on induced crystallization and x-ray diffraction analyses have revealed a remarkable similarity in the composition and structure of the calcareous corpuscles from tape-worms and that of the dense granules from mammalian mitochondria.

In a study of sterols, it was found that cholesterol occurs almost exclusively in its free form in the tissues of adult *T. taeniaeformis*.

Mechanisms of Energy Metabolism

Uncoupling phenols interact with the protein moiety of mitochondria, and thereby induce structural and functional changes. A comparison of the interaction of pentachlorophenol with mitochondrial protein, bovine serum albumin and myosin disclosed that free amino groups are involved in the binding of uncoupling phenols to these proteins.

Mitochondria uncoupled by pentachlorophenol exhibit a characteristic distorted morphology. Apparently only exogenous ATP can react with the contractile elements to restore normal morphology, whereas intramitochondrial ATP or high energy intermediates generated during electron transport are incapable of doing so. These studies demonstrate compartmentation of ATP within the mitochondrion, but do not show any clear correlation between biochemical function and structure.

The possibility that heart failure in man may be due to a bioenergetic defect in the myocardium which involves an impairment of oxidative phosphorylation was examined. Mitochondria were isolated from myocardial tissues at the time of cardiac surgery and examined for metabolic activity and by electron microscopy. The results indicated that electron transport and coupled phosphorylation were normal in these mitochondria, as was structure.

Malaria—Paralysis Factor

A previous report has mentioned the paralyzing factor for rats associated with strains of *Plasmodium berghei* which had undergone mouse passage. In collaboration with the Laboratory of Bacterial Diseases, the etiology of the paralysis has been elucidated. The joint work

is reported in the summary of LBD activities, page 7.

Toxoplasmosis and Sarcosporidiosis

Studies on parasitemia in the chronic infection in mice have shown blood infective for as long as five months post-infection. Tissue cultures infected with small numbers of relatively avirulent strains produce parasite forms resistant to peptic digestion, presumably cysts, as late as 23 days post-inoculation.

A study has been initiated on the occurrence of *Toxoplasma* in the heart and lungs of serologically positive patients coming to autopsy at the Clinical Center. One of 18 heart specimens was found infected with the parasite.

Sacrocystis muris fed to germfree mice did not invade the mucosa, and did not establish an infection in these mice nor in others that were fed their feces at various intervals. This experiment, confirming previous more limited studies, does not substantiate the route of infection postulated in early literature.

Immunologic Diagnosis of Parasitic Diseases

The same antigen used in the hemagglutination test for toxoplasmosis has proved satisfactory in a latex agglutination test, and agreement between the two tests is relatively good. The stability of the latex sensitized particles is much greater than that of similarly sensitized red blood cells.

Concomitant toxoplasmosis in leukemic patients has been demonstrated in a few instances. The latest case is that of a young boy with a space-occupying CNS lesion from which *Toxoplasma* has been isolated at biopsy.

A purified skin test antigen containing ribonucleoprotein has been tested in parallel with the usual toxoplasmin preparation and has been found equally effective in eliciting skin reactions in serologically positive human beings. A hemagglutination test was used to measure antibody levels in Puerto Ricans undergoing continuous, slow infusion of stibophen for schistosomiasis. Antibody levels at 4 to 6 weeks were 6 to 8 times higher than at beginning of therapy. If the increases in titer were stimulated by antigens derived from dead

worms, serology may be useful in assessing chemotherapeutic effects of drugs.

LABORATORY OF BIOLOGY OF VIRUSES

Although the essential emphasis of the program of this laboratory is on the basic mechanisms of virus replication and action, these are so intimately dependent upon and inter-related with the biochemical systems operative in the normal cell the virus infects that a fair proportion of our effort is concerned with strictly cellular events. Furthermore, in order to interpret any abnormalities caused by the virus there must be a firm foundation of knowledge concerning normal control cell performance.

Polynucleotide Homologies Among Viruses or Cellular RNAs and/or DNAs

Through the use of nucleic acid hybridization experiments, it has been shown that the messenger RNA's from different organs may be distinguished one from another although there are some basic similarities, whereas the DNA's from various organs appear to be identical. Although this has been assumed to be true based on biological functions, this is the first direct demonstration of the fact. Other studies of DNA from normal cells of different animal species has demonstrated a common "backbone" of base sequences between the DNA's of quite divergent species. The degree of "commonness" was directly correlated with the classical morphological classification relationships in a comparison of the DNA's from a series of sub-human primate species. For example, chimpanzee DNA is similar to human DNA that degrees of relationship cannot be distinguished by the direct competition reaction currently used. As we go down the scale of relatedness, gibbon DNA is 94% related to that of man, rhesus is 88%, the capuchin monkey is 83%, and the tarsier is 65%. An approximate 20% relatedness is characteristic of a large number of non-primate mammals.

As mentioned in the report of LBD, the characteristics of *Brucella* species DNA were studied. *Br. abortus*, *Br. melitensis*, and *Br. suis* are indistinguishable. *Brucella* DNA is very close to adenovirus DNA as far as purine content is concerned. Similar approaches are now

being used to compare the DNA's and messenger RNA's of normal versus virus-transformed tumor cells.

Physical and Chemical Properties of Virus and Viral Replication

Investigations of the physical and chemical properties of the virus particles themselves, both in their final mature infectious form and also in precursor stages of development, have proceeded along several lines. Early in poliovirus infection of human cells in tissue culture, viral particles are seen within cytoplasmic vesicles which appear to have arisen as protrusions of the nuclear membrane. The RNA associated with the specific protein-producing polyribosomes of poliovirus infected cells has been shown to have the base composition and size of viral nucleic acid. A replicative form of double- and perhaps triple-stranded specific poliovirus RNA has been characterized, and the presence of a strand complementary to viral RNA has been directly demonstrated. A continuation of studies on the multiplication cycle of vaccinia virus has demonstrated viral specific messenger RNA being synthesized in the cytoplasm of infected cells and together with associated viral protein synthesis is localized in polyribosomes. A newer approach to substructure of different viruses has been initiated by studying the penetration and binding of radio-labeled Actinomycin. Studies of the DNA's of polyoma and SV 40 tumor viruses by strand separation and reannealing as well as by sedimentation patterns strongly suggest complex super-structure in addition to the previously established circularity of these nucleic acid molecules.

Interferon Action Mechanisms

The role if interferon in suppressing viral infections of animals has been further defined by the demonstration of its production as a result of viremia. The interferon so induced not only reduces the viremia but protects the target organs from being seeded from the blood. A continuing study of the mechanism of action of interferon indicates that the viral inhibiting effect is operative at an early stage of virus

replication and may indeed be influencing the action of the input virus.

Viral Oncogenesis

Investigations of certain aspects of viral oncogenesis which have continued for several years in this laboratory have uniquely combined virological, immunological and oncological techniques. The original demonstration of new cell antigens in virus-induced tumors has been confirmed by many others and the phenomenon appears to be a general one. The subsequent demonstration of new complement-fixing antigens in transformed cells has broadened this aspect of virus tumors. Our studies have shown that the homograft type of virus-induced antigen is different from the CF type, yet both are specific and neither is represented in the structure of the virus particle.

Genetics of Animal Viruses

Three distinct plaque mutants of SV 40 virus have been isolated, purified, and compared in regard to their oncogenic capabilities. No correlation was found between *in vitro* and *in vivo* oncogenesis with these strains.

Conclusive evidence has been obtained that tumor cells previously transformed by one oncogenic virus, polyoma, can be "super-transformed" by SV 40 virus, as demonstrated by the presence of CF antigens.

LABORATORY OF INFECTIOUS DISEASES

The efforts of this Laboratory were focused on four major programs:

1. The viral and mycoplasmal causes and prevention of acute respiratory illnesses.
2. The role of viruses in cancer and leukemia.
3. The role of viruses, particularly of German measles (rubella), in the outcome of pregnancy.
4. The epidemiology of eosinophilic meningitis and picornaviruses in the Pacific areas.

The virus research groups in LID are working very closely with units in NCI and in NINDB in efforts to solve problems relating to the first three programs above, and are also

closely involved with the collaborative programs of three Institutes (NIAID, NCI, and NINDB).

Adenovirus Type 4 Enteric Capsule Vaccine

A new type of live virus vaccine which utilizes selective subclinical infection of the intestinal tract was shown in field tests with the U. S. Navy to be completely protective against adenovirus type 4, the most common cause of acute disease and hospitalization in military recruits. The vaccine was given orally in enteric-coated capsules that passed through the stomach unopened. The virus vaccine was released in the small intestine where infection was established, inducing protective antibodies in all of the recruits to whom it was given; yet the immunizing infection produced no illness and did not lead to spread or dissemination to non-immunized recruits.

The effectiveness of the adenovirus type 4 capsule vaccine has already been confirmed by other research groups.

Steps Toward a Respiratory Syncytial (RS) Virus Vaccine

Respiratory syncytial virus is the single most important cause of severe and fatal pneumonias in young infants. All attempts to date to make an effective vaccine against this virus, which is very unstable, have been unsuccessful. However, in recent months LID scientists have achieved biophysical characterization of the RS virus and its soluble antigens. Separation of the latter has led to the identification of one as the antigen which stimulates protective antibodies; this should speed the production of vaccines containing maximum amounts of this antigen.

Vaccine Against Primary Atypical Pneumonia (PAP)

A pilot lot of *Mycoplasma pneumoniae*, the cause of PAP, was shown to stimulate protective antibody in a significant proportion of individuals. This is the first time such evidence has been obtained. An important step in the evaluation of the *M. pneumoniae* vaccine was the development in the respiratory unit of a very sensitive new technique which can meas-

ure the specific growth-inhibiting antibodies in sera against all the known human *Mycoplasma*. This procedure not only made possible ready identification of *M. pneumoniae* but also of *M. hominis* type I which was shown to cause upper respiratory disease and pharyngitis in susceptible human volunteers.

Adenoviruses as Cancer-Inducing Viruses

Six human adenoviruses and several animal adenoviruses have now been shown to cause cancers in newborn hamsters, and in thymectomized rats and mice and to transform hamster, rabbit and rat cells in tissue cultures. These same viruses are known to cause a variety of widespread acute infections in their natural hosts, involving various parts of the body including the throat, eyes, lungs, liver, brain, intestines, and lymphoid tissues. It is essential that their possible roles in the induction of human cancer be assessed as soon as possible. LID's contributions toward this end in FY 1965 can be cited as follows:

Adenovirus type 3 was shown to cause tumors in hamsters. This virus has been reported from all parts of the world as the chief cause of epidemic pharyngoconjunctival fever and of many other respiratory and eye infections. Extensive surveys indicate that in the last ten years this virus has succeeded in infecting 60% to 70% of the populations of both hemispheres. If adenoviruses are found to cause cancer in man, type 3 would have to be regarded as the prime suspect and serological studies have been started to determine if type 3 adenovirus infections have occurred more frequently in cancer patients than in controls.

Neoantigens

Neoantigens are new virus-specific antigens recognized in cancer-inducing virus preparations and in the tumors they cause. These antigens were first reported by LID scientists as complement-fixing antigens in virus-free tumors of hamsters induced by adenovirus type 12 and 18. Similar antigens were subsequently demonstrated by Dr. Rowe's and Dr. Habel's groups in NIAID, and by others elsewhere in SV 40—and polyoma-induced tumors.

Studies of the neoantigens produced by adenovirus type 12 in infected tissue cultures revealed that some of them are produced very early after infection of the cell, that they are located in the nucleus (detectable by immunofluorescent procedures) and that they are not incorporated in the virus particle. It was determined that this is the same virus-specific but non-virion antigen which is produced in adenovirus type 12-induced tumor cells.

The discovery of the neoantigens has led to several important theoretical considerations as well as practical applications. They furnish one of the major focal points of debate on the mechanisms of cell transformation and neoplasia due to viruses. Their non-virion nature has led to questions as to whether or not they are coded for by virus or by cell genes.

For practical purposes they provide a virus-specific antigen by which the specific viral origins of tumors induced in animals but which contain no virus can be frequently recognized. Extensive systematic surveys for the presence of virus-specific tumor antigens in human cancers and/or antibodies against them in cancer patients are just getting under way.

Adenovirus—SV 40 "Hybrids"

Tests of an SV 40-free adenovirus type 7 seedstock, used in commercial vaccines, for oncogenic activity in newborn hamsters yielded numerous tumors having the neoplastic and antigenic characteristics of SV 40 virus; secondarily a few tumors also developed adenovirus type 7 antigens. The tumors appeared much earlier than those produced by SV 40 virus or adeno type 7 virus alone. Subsequent tests revealed that this was not a unique phenomenon but demonstrable in virtually all of the adenovirus type 3 as well as adenovirus type 7 vaccine seedstocks grown in rhesus monkey kidney; these seedstocks were subsequently cleared of detectable SV 40 virus, following which they were approved for use in commercial vaccines.

The fact that the adenovirus virion carries information capable of inducing tumors with specific SV 40 characteristics (in the absence of any vestige of the SV 40 virion) suggested that the information responsible for these ac-

tivities from the SV 40 virus must be incorporated in the adenovirus particle. All subsequent evidence has served to confirm this hypothesis.

Early studies designed purposely to produce additional adenovirus-SV 40 oncogenic combinations have led to evidence that the oncogenic activities of both groups of viruses can be stepped up enormously; thus when adenovirus type 12 and SV 40 are grown together, the resulting virus population induced lethal tumors in 25 days in 100% of hamsters; this appears to be the "hottest" oncogenic viral preparation available to date; the tumors have the virus specific tumor antigens of both viruses.

Studies of Leukemia Viruses

In vitro tissue culture detection and assay tests were developed and reported for all strains of avian leucosis and sarcoma viruses and for most of the known strains of mouse leukemia virus. These technical breakthroughs promise to make possible for the first time studies of the natural history of these viruses in their natural hosts. The COFAL test (complement fixation test for avian leukemias) has already been applied successfully to field studies of leucosis outbreaks in collaboration with the Regional Poultry Laboratory at Michigan State University, and the test is now widely used for diagnosis of both apparent and inapparent infections with leucosis and sarcoma viruses.

The mouse leukemia virus test is also based on the development of specific CF antigens in tissue culture infected with various leukemia viruses. It has been successfully used to isolate viruses from the tissues and embryos of strains of mice which have high rates of spontaneous leukemias. The test is *virion-specific*.

Both tests (avian and mouse) have been used in the search for virus-specific antibodies in more than 100 patients with human leukemia. Contrary to highly publicized reports from other laboratories, the results were completely negative. Since the LID *in vitro* tests are to our knowledge the most specific for antivirion antibodies, these tests seem to us to have a degree of validity not demonstrated for the other tests, which suggested that human and animal leukemias viruses are antigenically related.

Surveys of Human Cancer Patients for Evidence of Virus Induction of Their Cancers

Over 700 cancer patients and controls have been surveyed for evidence of virus-specific tumors, viral antigens and neoantigens in their tumors, and for antibodies in their blood sera. These surveys are only beginning. However, a number of abnormal distributions of viral antibodies have been noted in certain categories of cancer patients. These possible leads will be explored more fully when larger numbers of properly controlled specimens can be obtained.

Collaborative Studies with NINDB on the Role of Infectious Agents in Perinatal Disease

This program will be mentioned only briefly here, since the work will be reported in detail by NINDB, which provides the funds for most of the work. The laboratory project is housed in LID, NIAID, and receives its scientific direction from the Chief, LID.

The principal accomplishments this year have been the development and exploitation of a new rapid complement fixation test for rubella infection and the demonstration of persistence of rubella virus in congenitally infected children for at least 6 months after birth. Data on the occurrence of salivary gland virus, a large number of other viruses, and *Toxoplasma* have been collected for analysis.

Studies on Eosinophilic Meningitis and its Cause, *Angiostrongylus cantonensis*

Epidemiological and laboratory studies of eosinophilic meningitis in man were continued, and over 1,000 laboratory-proven cases are being analyzed. All the data suggest that the food habits of certain populations in French Polynesia and on other Pacific islands are responsible for the cases. The evidence still points to the consumption of raw crustacean intermediate hosts of the rat lung parasite *A. cantonensis*, as the prime source of human infection. In man the larvae do not invade the pulmonary arteries but instead locate in meningeal areas of the central nervous system. Certain pelagic fish suspected on epidemiological grounds to be the cause of cases of eosinophilic meningitis in Tahiti were fed third stage *A. cantonensis*; the latter survived and migrated to the muscula-

ture, thus suggesting that fish could indeed be the source of human infection when eaten raw.

Virus Infections in the Pacific

Studies of picornaviruses led to the discovery of new ECHO virus serotypes 29 through 32. Picornaviruses have been demonstrated in 12% to 15% of 2500 specimens collected from persons living in the Pacific area.

In 1964 reports suggested that the reoviruses of mammals were in many ways identical to the wound tumor virus of plants. Serological studies showed they were not related immunologically and biological studies showed that three serotype strains of reovirus would not multiply in leaf hoppers, which are the vectors of the wound tumor virus.

Inactivated Measles Vaccine Test

An evaluation of an inactivated measles vaccine was carried out. The vaccine was given in three doses at 14-day intervals; no serious reactions were encountered. An outbreak of measles occurred two months after the final injections of the vaccine. Ten to thirteen percent of the children given inactivated vaccine developed measles compared to 77% to 79% of unvaccinated children. This study established the fact that the inactivated vaccine would produce a profound effect on a pending measles epidemic, provided the vaccine was given within a few weeks or months of the outbreak.

Electron Transport System in an Autotrophic Hydrogenomonad

The initial step in oxidation of molecular hydrogen to water by *Hydrogenomonas eutropha* was previously shown to be transfer of electrons from hydrogen to the acceptor, nicotinamide adenine dinucleotide (NAD). This year, three possible pathways for electron transfer from the reduced acceptor, NADH, have been delineated. In one, an enzyme (menadione reductase) mediates electron transfer to Vitamin K3 (menadione). The enzyme, purified 15-fold, has been studied in detail. The reduced menadione appears to be able to reduce cytochrome c directly, without enzyme mediation. A second enzymatic route of electron transfer is to cy-

tochrome c by means of cytochrome c reductase, and thence to cytochrome c oxidase. A third route, probably enzymatic although clearly unphysiologic in the experimental system employed, allows electron transfer to, and reduction of, ferricyanide; the natural hydrogen acceptor for this route is as yet unknown. This electron transport system in *Hydrogenomonas* is of special interest because it is non-particulate except possibly for the last step.

Biochemical Studies on Staphylococcal Cell Walls

Previous studies have shown an enzyme in *Staphylococcus aureus* which decreased cell wall density. It was postulated that the released material might be teichoic acid. In the past year, it was found that some of the enzyme was bound to native cell walls. The enzyme has been partially purified and its optimal activity determined. Bound enzyme differs from soluble in its pH range and optimal activity in the absence of salt. Molecular sieving experiments suggest that the material it releases is a polymerized teichoic acid. Of primary interest, and yet to be determined, is the role of this "autolytic" enzyme in staphylococcal metabolism, particularly as a possible "uncoupler" to allow incorporation of newly synthesized components during cell wall biosynthesis. Understanding of such mechanisms seems essential to solution of problems of antibiotic resistance and modes of action of some classes of antibiotics.

Iron and Siderophilin in Metabolism of Bacterial Pathogens

The importance of iron in growth and metabolism of *Staphylococcus aureus* was investigated, using a newly developed method for removal of iron from a complex growth medium. Relative iron deficiency caused loss of Krebs cycle activity and a decrease in glycolytic activity. When grown in normal human serum, the same strain of *Staphylococcus aureus* failed to produce coagulase, hyaluronidase, hemolysins, or staphylokinase, although producing them in the usual media. Saturation of the serum with iron resulted in production of coagulase, though not of the other putative pathogenic

factors. In preliminary studies, the effectiveness of several antibiotics against the test organism growing in serum was shown to be related to the serum iron level and the percent iron saturation of siderophilin.

Pathogenic Fungi

Habitat studies of pathogenic fungi have been continued at sites studied over a period of years, in new sites associated with cases of mycotic infection, and at ecologically interesting sites. Critical examination of soil profiles has shown that, contrary to a recent report, *Histoplasma capsulatum* grows in only very superficial layers of soil.

Taxonomic problems involving the dematiaceous fungi which cause subcutaneous ulcers, mycetoma and chromoblastomycosis have been elucidated. Studies were stimulated by a 6 weeks' trip for World Health Organization in Europe and Africa where the current incidence and medical and public health importance of mycetoma, North American blastomycosis, subcutaneous phycomycosis, and rhinophycomycosis, were surveyed.

Physiologic studies of three strains of *Coccidioides immitis*, comparing the metabolic activities of the hyphal and spherule forms of this pathogenic fungus, show that in cell-free extracts of the spherule form, citogenase, isocitric dehydrogenase and isocitric lyase activities are significantly less than in cell-free extracts of the hyphal form. When the effect of decreased concentration of nutrients in the medium used for spherule-production was critically tested, the activity of isocitric dehydrogenase of the hyphal form grown in dilute medium declined to the level in spherules, while the activities of the other enzymes decreased only slightly.

Antifungal Agents

Fourteen drugs and antibiotics were tested for antimycotic activity by the tedious but informative methods of mouse infection and experimental chemotherapy. Among these, only Hamycin merits clinical trials. We have tested its effect as different doses and in combination with amphotericin B, investigated its toxicity, and developed a bioassay method for serum lev-

els. It is effective when given by mouth or intraperitoneal injection in preventing death and in sterilizing infected organs in mice with experimental blastomycosis, cryptococcosis, histoplasmosis and candidiasis. Bioassay reveals levels of Hamycin in dog sera and human sera and pus at levels sufficiently high to exert an effective antifungal activity. Attempts have been continued to arrange for production of X-5079C.

Mycotic Infections

A vaccine, prepared by exposure of spherules to hydroxylamine, protected mice against challenge by spherules of *C. immitis*, but was no more effective than immunization with formalin-killed spherules.

Studies of the structure and antigenic properties and determinants of species of *Candida* and other fungi have been continued. Using mannans prepared from two strains of *Candida albicans*, it was demonstrated that mannans with identical linkages may react differently to antisera depending upon the degree of branching of the polysaccharides. The major antigen on the surface of yeast cells of several species of *Candida* is mannan. Cross reactivity of *C. albicans* mannan with *Salmonella typhosa* antisera indicates similar mannosidic linkages in *Candida* and *Salmonella* polysaccharides.

Antimicrobial Agents

In continuing investigations of antimicrobial agents from molluscs, two methods for extraction and isolation of active material from clams were used. The substance obtained inhibits herpes simplex growth in tissue culture as well as the development of adenovirus 12-induced tumors in young hamsters. It is of low molecular weight (700-1500), heat-stable, water-soluble, non-toxic, and contains about 5% nitrogen.

Bacterial Structure and Function

Previously developed methods of immunofluorescent labelling have been applied to study of cell-wall replication in a variety of bacteria. *Escherichia coli* replicates wall in a diffuse intercalary fashion, comparable (as anticipated)

to that previously shown in the related *Salmonella typhosa*. The mode of cell-wall replication in the genus *Bacillus* is unclear.

Continuing studies on adsorption of temperate streptococcal bacteriophage have limited by exclusion what the cell-surface receptor is not, but have not defined precisely what it is. It is not the hyaluronic acid capsule, the group-specific polysaccharide, nor the type-specific proteins M nor T. It does appear to be an antigenic surface component which is labile to aging, heat, chloroform, and ultraviolet irradiation, while resisting trypsin and pepsin.

The developmental cycle of a Group C streptococcal bacteriophage in its host cells has been followed by electron microscopy of ultrathin sections.

ROCKY MOUNTAIN LABORATORY

Management of the research program has continued under the dual mission concept which serves to provide broad objectives and guidelines to projects. Through a functional reorganization of the laboratory designed to weld units with common purpose and interests into working groups, progress has been made in the more efficient use of personnel, equipment and facilities. The laboratory continues to interest scientists, both here and abroad. More than 40 foreign and resident visitors were received and 10 guest workers (four foreign) were hosted and supported in various units. Three NIH-supported fellows pursued their studies here last year and plans have been made to accept a few additional fellows from many young interested graduates.

Tularemia

Sustained interest in tularemia is imperative in view of the rather severe outbreaks of disease this year. During the active tick season in 1964, tularemia was a major economic problem to sheepmen, both in livestock losses and human disease in Garfield and Rosebud counties in Montana. Of 18 human cases reported, 15 occurred among persons closely associated with the sheep industry. Two outbreaks on beaver farms in Utah and Washington were investigated.

Continued studies on immunoprophylaxis against tularemia revealed that the protection engendered in mice by a single dose of the living Russian vaccine declines with time and is sufficient after one year to protect only against strains of degraded virulence. Mice vaccinated with killed vaccines (cell wall preparations) are protected only against weakly virulent strains and this immunity cannot be increased by additional doses. Although this vaccine is weakly immunogenic, it is useful as a skin-test antigen. In an epidemiologic survey for tularemia among trappers, 17 percent reacted. The skin-test positive rate was directly related to age and to the intensity and duration of trapping activities; the rate among trappers who had taken 500 or more pelts annually was 30 percent. Generally, the size of the skin reaction was not affected by the virulence of the infecting strain or the period since infection.

Rabies

Studies on the spontaneous recovery of mice from rabies have been continued, partly because of its intriguing relationship to the possible occurrence of chronic rabies in man and carrier status in vector species. In disease induced by intraperitoneal injection of virus, the virus content of the brain was not as high, nor did it tend to increase with continued survival, as it did after intramuscular or intracerebral inoculation of virus. Virus disappears about 7 days after onset, coincidentally with a rather sudden virus neutralizing capacity of brain tissue. However, serum neutralizing antibodies appear much earlier and coexist with brain virus even before onset of illness. Mortality after subcutaneous injection of virus in the nuchal area was surprisingly lower than that observed when virus was deposited at the base of the tail. When virus was given at the nuchal site, the mortality was comparable to that seen after intraperitoneal inoculation, but the latter route produced a much higher incidence of abortive infection.

Rickettsial Diseases

Further study of the ecology of *Rickettsia rickettsi* in western and eastern Montana has

yielded additional information that suggests each of the types (R, S, T, U) have unique ecologies. For example, Columbian and golden-mantled ground squirrels and chipmunks developed significant rickettsias for eight days when they were infected with the fully virulent R type. However, these animals were completely refractory to the T and U types. Likewise, *Dermacentor andersoni* females transmit rickettsias of the R types to 100 percent of their progeny but they transmit the mild *Haemaphysalis leporispalustris* (Hlp) rickettsia to only 50 percent of their progeny.

The M rickettsia, which was initially recovered from *Microtus* sp. and *D. variabilis* in eastern Montana, was isolated from 10 percent of 90 *Peromyscus* sp. collected in the same study area. Of mice taken in June, 28 percent were seropositive. This year the M organism was recovered also from 10 percent of *D. variabilis* taken in widely separated areas in Nebraska and North Dakota. Conceivably, the prevalent M organism may interfere with the ecology of the virulent T type and prevent its occurrence in the Midwest in the same species of tick that transmits the agent to man on the East Coast.

Studies on the role of domestic and feral animals in the epidemiology of typhus were expanded in Egypt and extended into South America. A staff scientist has been assigned to NAMRU-3 to coordinate two projects on this problem supported by PL480 funds. To date, results of serologic surveys in Egypt confirm earlier observations of typhus antibodies in serums of livestock but the type involved has not been determined. Of 17 positive goat and camel serums received for confirmatory testing, four reacted specifically with *R. prowazekii* and four with *R. typhi*, while nine reacted alike with both types.

With travel supported by PAHO, two staff scientists made a 3-week survey of typhus in Ecuador to determine whether domestic animals and ticks are involved in the epidemiology of the disease. Of 26 serums obtained from persons who had typhus within the past five years, 22 had typhus antibodies. Most of these serums reacted chiefly with *R. prowazekii*, and the remainder reacted equally with both typhus rickettsias. Except for 5 of 8 donkey serums which

were positive, the rest of 387 serums from cattle, sheep, goats, and pigs were negative. Apparently, domestic animals are not as extensively involved there as they are in Egypt.

Transmission of Disease

Although the entomological staff have re-oriented much of their research program, they have continued to honor requests for taxonomic support from various organizations. In connection with MARU's study of Bolivian hemorrhagic fever, 500 specimens representing those tested for BHF virus were identified. The taxonomy of the ornithorine tick larvae of the world was recently completed. Incidental to these and other services, specimens belonging to three new genera and 33 new species were described in publications.

Various media and modifications thereof and a wide variety of handling methods were used in attempts to establish continuous tissue-culture cell lines from arthropod tissues. About 2500 cultures were prepared from tissues from nine species of ixodid ticks; although primary growth from explants was readily obtained, maintenance of a continuous cell line has not been achieved.

Investigating acquired resistance of mice to louse infestation, it was found that heavy and constant exposure was required to induce resistance. Resistance was limited only to the site previously infested. Strains of mice varied not only in their innate susceptibility, but also in their ability to acquire resistance after infestation.

In studies on the heritability of the paralytic factor in *D. andersoni* ticks, the proportion of paralytic females has, with one exception, remained constant through six generations.

Encephalitides and Other Tick-Borne Diseases

In the final phases of an ecologic study of Colorado tick fever (CTF) and Powassan virus in South Dakota, it was shown that the important CTF nymph-small-mammal-nymph or larva cycle is active chiefly during May through August. *D. andersoni* was the major vector of CTF whereas *Ixodes spinipalpis* was the chief vector of Powassan virus. Since the latter species rarely bites man, and since Po-

wassan virus is rarely found in *D. andersoni* which frequently feeds on man, Powassan disease will not likely emerge as a public health problem.

This year CTF virus was isolated from 40 patients whose time of illness and residence corresponded with the seasonal activity and known range of *D. andersoni*. A sufficient number of cases are now documented to conclude that severe involvement of the central nervous system or hemopoietic system is uncommon.

In the field study areas in Bismarck, North Dakota, and Vale, Oregon, little activity of WEE and SLE was observed. Neither virus was recovered from over 4,000 *Culex tarsalis* collected and all sentinel chickens remained seronegative during summer.

Psittacosis-Lymphogranuloma-Trachoma (PLT) Group

Major efforts in this project were directed toward developing serologic and immunologic methods of identifying members of the group and specific antibody responses they induce in various hosts. So far the radioisotope precipitation (RIP) test has shown most promise. In this test, meningopneumonitis virus cultivated in phosphorus-depleted mouse "L" cells and labelled with P³² is used as a test antigen. Although its sensitivity is somewhat limited by nonspecific precipitation of antigen, the RIP test appears to be far more sensitive than the complement-fixation (CF) test. Thus, four virus-positive patients with trachoma and one treated patient were RIP seropositive whereas two patients having phlyctenular keratoconjunctivitis were RIP negative. All seven were devoid of CF antibodies to a number of PLT antigens. Other persons with frank cases of psittacosis have shown antibody rises detectable by both CF and RIP tests.

An outbreak of trachoma occurred among Indians at schools at Busby and St. Labres Mission, which apparently stemmed from one family with the appropriate name of "Wounded eye." After five students from this family had eye infections during the 1963-64 winter, eye infections increased noticeably in the student body, particularly among close associates of Woundedeye children. Some infections among

teen-age girls were apparently transmitted by the common use of mascara brushes. From specimens collected during the investigation, trachoma-inclusion conjunctivitis (TRIC) agents were recovered from four Woundedeye children and one roommate of a Woundedeye child. As revealed later by ophthalmologic examination, 22 percent of 241 students at the Busby school had lesions suggestive of early trachoma whereas only four percent of 260 students at the St. Labres school had such lesions.

Chronic Progressive Viral Disease

Comprehensive studies on four animal diseases, scrapie and intersititial pneumonitis of sheep and subacute encephalopathy and Aleutian disease of mink, are underway to provide basic information for the selection and study of human diseases which conceivably have comparable pathogeneses.

From the 2½ year study on the pathogenesis of scrapie in mice just completed, additional data sustain previous observations that pronounced early growth of virus occurs in many extraneural tissues. Preliminary observations of scrapie in goats indicate a different pathogenesis because virus has been limited chiefly to the nervous tissue (brain, spinal cord, and sciatic nerve) and embryologically related pituitary gland and adrenal gland in the few goats inoculated in 1962. Information obtained from the long-term pathogenesis study in goats started in October 1963 supports this conclusion also. To date virus has been recovered only from the left prescapular lymph node of a goat killed 24 weeks after subcutaneous inoculation behind the left elbow. From tests so far completed, virus has not been detected in the brain for ten months after subcutaneous inoculation.

In Aleutian disease of mink, virus was detected through the 10⁻⁵ dilution, the highest tested, in mesenteric lymph nodes, intestine, spleen, serum, and heart and through 10⁻⁴ in liver, kidney, and lung. Noteworthy is the large virus content of serum, despite high levels of circulating gamma globulin. This virus, in 650 m_μ millipore filtrates of the 10⁻² dilution of a tissue suspension, was unaffected when subjected to 56°C. for 30 minutes, slightly inactivated at 60°C. and markedly inactivated at 80°C.

The virus of encephalopathy of mink, a new disease of ranch-bred mink discovered last year, has been transmitted to Sapphire, Pastel, and Pearl mink inoculated intraperitoneally with crude and filtered (300 m μ millipore) brain and spleen suspensions from a naturally affected Idaho mink. So far the incubation period has varied from 6½ to 11 months.

Allergy and Immunology

Staff scientists found that thymectomized mice died within ten days after inoculation with doses of *Candida albicans* which normal controls cleared from their tissues within eight days. Thymectomized mice also were more vulnerable to endotoxins from Gram-negative bacteria than were normal controls and repeated doses of *Salmonella typhosa* endotoxin accelerated post-thymectomy "wasting."

Preliminary studies were made of the dermal reactivity of mice to evaluate their usefulness in allergology. Although others have reported that mice develop delayed hypersensitivity, only Arthus-type reactions to large doses of HEA could be produced. The antigen had to be mixed with Freund's adjuvant and at least 30 days had to elapse before sensitivity could be elicited. In the measurement of antibody response in mice, the Boyden hemagglutination test was studied to improve its specificity. Saline-diluted mouse blood was shown to contain residual fibrinogen which nonspecifically agglutinated tanned sheep red cells.

Immunoprophylaxis Against Tuberculosis

After repeated attempts to prepare a killed vaccine against tuberculosis, RML scientists discovered that the cell walls derived from a moist mass of Mycobacteria processed in oil through the Ribi press were immunogenic. Progress from this original lead had been gratifying. Not only have subsequent studies confirmed original observations, but also the cellular site of the protective antigen has been identified and means developed to improve the potency of the vaccine.

To date the most potent vaccine has been prepared from cell walls derived from *Mycobacterium tuberculosis* suspended in saline when disrupted in the Ribi press. The cell walls are lyophilized, mixed with just enough miner-

al oil to make a smooth paste (1 to 2 drops/50 mg cells) and then the oil-treated cells are suspended in 0.85% sodium chloride solution containing 0.2% Tween 80.

The protective antigen appears to be an inner component of the cell wall. In an experiment designed to locate the protective antigen, it was shown that killed whole BCG, with or without oil, and protoplasm were not immunogenic. Only cell walls treated with oil proved to be effective vaccines. Immunogenic products extracted with organic solvents to remove the oil lost their potency, but it could be restored merely by adding oil again to the dried cells. Thus, it appears that the protective fraction on the inner cell wall must be exposed and coated with oil in order for the vaccine to be protective.

The relationship between potency of oil-treated cell walls and its sensitizing ability to PPD has not been resolved but preliminary studies indicate that the ability of killed vaccines to sensitize guinea pigs to PPD is markedly reduced. Conceivably, if all the protoplasm could be separated from the cell wall, a potent nonsensitizing vaccine should be possible.

Bordetella Pertussis Antigens

The development of methods of obtaining a chemically pure protective antigen, which so far has not been separable from the histamine-sensitizing factor (HSF), has received continued attention. This fraction has been isolated by Sephadex filtration and starch block electrophoresis, but the final material was heavily contaminated with substances from the system. Practically all the HSF activity was isolated from crude preparations by magnesium sulphate precipitation, but the active material derived therefrom was poorly soluble in saline. Nevertheless, the latter procedure is sufficiently promising to warrant an attempt at producing large quantities of HSF necessary for chemical characterization and for vaccine trials against whooping cough.

A major effort was made to elucidate the mechanism whereby HSF renders mice more susceptible to histamine. An increased sensitivity occurred within 90 minutes after mice were inoculated with HSF and persisted for at

least 84 days. Since the change in sensitivity is similar to that resulting from adrenalectomy or adrenal-demedullation of mice, the adrenal gland could conceivably be involved in the mechanism of action. Histamine caused a marked dilation of blood vessels in the ears of both normal and HSF-treated mice; 1-epinephrine restored the normal size of vessels in untreated mice but not in HSF-treated mice. Thus, it appears that HSF somehow interferes with the action of epinephrine.

Endotoxins

The molecular dimensions and weights of endotoxin, native haptene, and acid haptene were compared to gain some insight into the structure of endotoxin. Respectively, their weights were estimated at 17 million, 130 thousand, and 12 thousand. From axial ratios obtained by applying Einstein's viscosity increment, the length and diameter of endotoxin was 6800 Å by 75 Å, whereas those of native haptene and acid haptene were 1490 Å by 13 Å and 290 Å by 10 Å, respectively. Chemically, native haptenes contain the same major sugar components found in the homologous endotoxin, but they lack heptose, 2-keto-3-deoxyoctonate (KDO), phosphorus and long-chain fatty acid. Hence, from the preceding observations, native haptene would appear to consist of a single chain with space for very short-chain side branches. Probably it consists of trisaccharide of tetrasaccharide units. It seems likely that failure to combine native haptene units into fully active endotoxin is due to our inability to incorporate missing ingredients, which conceivably provide cohesive forces and modify the character of micelles formed from carbohydrate chains.

Morphologic Elements of Microorganisms

The ultrastructure of bacterial spores, particularly the exosporium, and the chemical, biological, and structural properties of bacterial L forms and their parent bacteria received primary attention. The exosporium of *Bacillus cereus* appeared to consist of two layers. The outer layer was composed of lead-stainable hairlike projections about 250A deep, which arose from an intermediate covering about 60A

in depth. An inner basal layer, which had a hexagonally perforate surface pattern, was composed of four lamellae, which could fragment into crystal-like elements. This crystal-like nature of the basal layer, as confirmed by X-ray diffraction, was shown to correspond to a hexagonal, closely packed crystal structure. The exosporium of bacterial spores is now recognized as the primary physiologic barrier between the spore and its environment and as a specific spore component rather than a sporangial remnant.

MIDDLE AMERICA RESEARCH UNIT (MARU)

The past year reflects significant further progress in the scientific program conducted at MARU. It also recorded many changes in staff and physical facilities.

Hemorrhagic Fever

Chronic Machupo virus infection with viraemia was thoroughly documented in hamsters and more importantly in experimentally inoculated *Calomys callosus*, the suspected reservoir in the San Joaquin, Bolivia, epidemic. Additionally, virus was isolated from urine of naturally infected *Calomys* in San Joaquin. A rodent control campaign, primarily aimed at *Calomys*, was carried out in San Joaquin with spectacular, rapid and complete interruption of the human epidemic. These data, together with continued inability to recover the virus from arthropods, leads to the belief that the disease was somehow transmitted directly from *Calomys* to man. A practical virus plaque neutralization test was developed and is now in use in sero-epidemiological studies.

Arbovirus Studies

Although field work was limited last year, VEE activity at Gamboa, Canal Zone, was again demonstrated, and two further isolations of EEE virus were made. The data suggest that these agents are endemic or regularly introduced into this area and provide further justification for more detailed future investigation.

Two new arboviruses, Changuinola and Chagres, were characterized and registered in the Arbovirus Catalogue.

Respiratory Virus Studies

Extensive sero-epidemiologic work at Paraiso, Canal Zone, was completed. Paired sera from more than 300 persons were analyzed. There is now ample documentation that infection patterns of a large number of viruses are similar in tropical and temperate areas. Differential communicability of rhinovirus serotypes was also elucidated for the first time anywhere.

Histoplasmosis

Additional genera of bats were found to be infected with the fungus. Documentation of fecal excretion of *Histoplasma capsulatum* by several species of bats was strengthened and, investigations into the physiologic dynamics of the phenomenon were begun. A large study of patterns of human serologic response to *H. capsulatum* infection was carried out. Results showed conclusively that utilization of both complement fixation and gel-precipitin techniques increases diagnostic efficiency.



NATIONAL INSTITUTE OF MENTAL HEALTH

INTRODUCTION

The summaries of the Laboratory and Branch Chiefs which follow this introduction give a good account of the research achievements of this program during the year which is just ending. It has been a good year and a productive one. Again, it has been our good fortune that, with few exceptions, our middle and senior scientific staff have stayed with us. Turnover of research personnel on the permanent staff has been remarkably low. We are proud of this even though the expanding needs of this maturing scientific staff for space and supporting personnel have us often at wit's end trying to squeeze out a spare half-module. We are hopeful that the new buildings now being planned will provide space relief within the foreseeable future.

Present estimates of completion dates are: February, 1968, for the NIMH-NINDB basic research building (Building 36); January, 1967, for the two laboratory buildings at the Poolesville Farm; and August, 1967, for the new Child Research Building. The process of completing plans for the last named has been complicated and difficult, and the staff of the Child Research Branch, now temporarily located in Wilson House, deserve our admiration for their steadfastness and courage in the face of a most frustrating search for a new headquarters.

A request for funds to plan a new building for the Addiction Research Center, to replace the present space in the basement of the Lexington Hospital, has been included in the preliminary 1967 budget request. In view of the amount of public interest in the problems of drug abuse and the difficulty of the scientific problems involved, it is important that new and better research space be available as soon as possible.

It is pleasant to record an event of recent months that represents an improvement in one

system of bureaucratic controls. The Federal Reports Act of 1942 requires Budget Bureau clearance of research projects which involve asking questions of people who are neither patients nor Government employees. Such research is done by at least half of our laboratories. All proposals for such research have had to be cleared by the NIH, the PHS, the DHEW, and the Bureau of the Budget; each echelon could take as long as it wishes with no explanation; the investigator at times had to answer the same questions from two or more people; the resulting delays at times forced the cancellation of projects; and the emotional wear and tear on investigators was severe. Some two years of negotiations have now resulted in an agreement by all echelons that the technical review of all such research projects coming from the NIMH Intramural Program shall be delegated to a Technical Review Committee, to be appointed by the Associate Director for Intramural Research from among the NIMH Intramural Staff. Policy review remains a prerogative of each higher echelon, but the agreement is that such questions must be raised within ten working days after technical review and approval, or an official Budget Bureau number is issued. The new system is on trial for a two-year period, and will be reevaluated at the end of that time.

Two things are crucial to the success of the new system: a careful and competent job by the Technical Review Committee, and the wholehearted cooperation of clearance officers at PHS, HEW, and BOB. Of the former we are assured. Dr. Richard Q. Bell has agreed to serve as Chairman of the Committee, with the following able colleagues: Dr. John D. Campbell, Dr. William Pollin, Dr. Morris Rosenberg, Dr. Earle S. Schaefer, Dr. Robert S. Shellow, and Dr. Samuel Greenhouse. A more competent committee could not be put together anywhere. As to the cooperation from the higher echelons,

there are encouraging signs, including the very gratifying good will evidenced during the negotiations. We shall hope for the best. The payoff can be not only a very significant improvement in the research environment for our behavioral scientists but potentially also a useful model for other agencies engaged in such research.

The past year saw four senior staff members working away from Bethesda. Dr. David Rosenthal divided his year between Israel and Denmark, initiating projects in both countries to investigate genetic factors in the etiology of schizophrenia. Dr. Melvin Kohn was in residence at the Institute for Social Research at the University of Oslo, Norway, studying and planning future programs. Dr. Morris Parloff utilized the facilities of the Institute for Personality Assessment and Research at the University of California, Berkeley, to continue his studies in creativity. Dr. Giulio Cantoni spent the first six months of 1965 at the University of Palermo, Italy, and at the Institute de Biologie Physico Chemique in Paris continuing his research on ribonucleic acid.

The NIMH Board of Scientific Counselors met twice during the period under review. On October 29 and 30, 1964, under the Chairmanship of Dr. Leonard Cottrell, the Board spent two half-days hearing research reports on (a) human psychological development, from the Child Research Branch, Laboratory of Socio-environmental Studies, Laboratory of Psychology, and the Adult Psychiatry Branch; and (b) molecular neurophysiology, from the Laboratory of Neurobiology, Laboratory of Neurophysiology, Laboratory of Clinical Science, and the Clinical Neuropharmacology Research Center. Time was allocated also for informal visits to a number of laboratories and for an executive session. On March 11-12, 1965, the entire meeting was devoted to a review by the Board and senior staff of suggestions for expansion of the Intramural Program when additional space is available. Dr. Howard Hunt served as Chairman in the absence of Dr. Cottrell. Staff committee reports on the following research areas were presented and discussed: Physical Biology (Giulio Cantoni, Chairman); Neurochemistry (Seymour Kety, Chairman); Psychopharmacology (Edward Evarts, Chair-

man); Biology and Behavior (Lyman Wynne, Chairman); Learning (David Shakow, Chairman); and Genetics (James W. Maas, Chairman). The discussion was animated, argumentative, often without a resulting consensus, and always helpful to those of us responsible for determining program directions. Members of the Board this year were Dr. Leonard Cottrell, Chairman, Dr. Howard F. Hunt, Dr. Merton M. Gill, Dr. Robert S. Morison, Dr. Heinrich Waelsch, and Dr. S. Bernard Wortis. To them all, as every year, goes our appreciation and gratitude for their interest, counsel, and friendship.

CLINICAL INVESTIGATIONS

The period that has passed since the last report has been in most respects a gratifying one. The reports of the several branches and laboratories reflect substantial achievements in every area of major concentration. Our senior investigators are invited participants at important national and international conferences and, as in previous years, members of the staff have won national awards from their scientific colleagues in recognition of the outstanding merit of their contributions. Some of the problems we face are touched upon in the comments of the laboratory and branch chiefs. The ever-present ones are the relatively inadequate salaries, the annual budgetary increments which do not take fully into account the steadily increasing costs of research and the moderate but still irksome restrictions on travel and some forms of research which seem to characterize governmental institutions but not private universities. However, the most crucial issue we face is internal rather than external. The next three years will bring us a sizeable increase in space and resources; a new building for the study of children and their families, a laboratory building to which some of the groups not engaged in research on patients will be transferred, and several buildings on the NIH farm for the study of certain aspects of animal behavior. The decisions we make as to the use of these facilities will largely determine the direction our program takes for the next ten years, and will be a limiting factor on the nature and extent of possible achievements.

The first step taken in planning for the best use of these new resources was to appoint staff committees to consider the areas of physical biology, neurochemistry, psychopharmacology, behavior and biology, learning and genetics. The reports of these committees have been discussed in detail by the branch and laboratory chiefs at their regular meetings, and also at a meeting with the Board of Scientific Counselors. There remain a number of issues to be weighed before the final decision is reached, hopefully in the coming year.

There is general agreement in defining our task as that of maintaining and developing even further an exciting, dynamic program—one which continues to have relevance to the important ideas current in the field as well as to those we believe may be just over the horizon, and one in which the productivity of the individual segments of the total program is strongly supported by the unique assets of the Clinical Center.

It is impossible to choose between the reports on the basis of substantive content alone. Each of the areas covered is of undoubted importance; each could be powerfully exploited in this setting. The arguments and considerations put forth by each committee are most persuasive. This is not surprising since the committees numbered among their members investigators who have earned international reputations and respect as leaders in their field; their considered judgments as to how best to support further development of their areas of special interest and competence are bound to command the most thoughtful and serious evaluation. Indeed, from one point of view, it might be felt that we could select almost at random any of these areas for successful exploitation. The importance of the decision probably lies not so much in the merit of the research a new group might accomplish as it does in the degree to which it might notably enhance the work of existing branches and laboratories.

As has been pointed out in earlier reports, the Clinical Investigations program was always envisaged as an interdisciplinary, but never as a completely comprehensive program of behavioral research. Since it was basic and

long-term rather than directed and targeted in character, its development was somewhat opportunistic, depending on the recruitment of the most competent investigators available in any of a number of areas rather than in a narrowly selected range of problems. We were most fortunate in having brought to the Institute a number of outstanding senior investigators, and were equally fortunate in enlisting the interest of a number of younger scientists who came relatively early in their scientific careers and whose major contributions have been made here. As the members of this group moved from their early research projects toward the development of research programs, we tended to divide our limited potential for expansion more or less equally, giving each of the several individuals and groups an increment needed to increase their strength, but most often not to a degree great enough for them to develop their plans fully. A portion of the new resources probably should be devoted to strengthening certain existing programs so they may reach a state of maximal productivity.

A second question concerns the imperative need for flexibility. At present we have none; our resources and space are fully committed; if a challenging new opportunity should present itself, we could pursue it only by discontinuing an activity in which we are already engaged. Often this can not be done in less than one or even two years. Yet there is not a branch or laboratory in the program which does not have ideas which merit exploration but which are yet too tentative to warrant the extended attention of permanent staff. To a degree the provision of a limited amount of unassigned space and personnel is the responsibility of the laboratory itself; a degree of freedom, although perhaps not absolutely essential, would greatly enhance the richness of a full-time research life, and it should be built into every program. But it is increasingly apparent that Clinical Investigations as a whole should maintain a small reserve which can be assigned for limited periods for the exploration of new ideas, or for the purpose of starting a new program while an old one is phased out. It is difficult in our present system to maintain vacant space and

unfilled positions without losing them, but in my opinion they are necessary for the health of a research organization.

Yet another question which deserves consideration is that of building or maintaining a critical concentration of investigators in each of the disciplines represented in our program. It is generally believed that the presence of such a critical mass is essential for optimum productivity. On superficial examination, the balance here would seem to favor assigning a major portion of the new space to a social science program, since NIMH is presently the only Institute in which there are a significant number of investigators in that area, while the biological sciences are strongly represented in every Institute—so much so that not altogether jokingly the Clinical Center has been described not as a hospital but rather as a biological institute with beds. Perhaps the critical issue here is not just the relatively lower concentration of social and behavioral scientists, but whether a moderate increase in facilities for biological studies would exponentially strengthen the existing laboratories by filling in important defects in our coverage of the field.

Finally, the next ten years will see the retirement of some who started this program. For the first time some will have to consider not only the studies we plan to do ourselves, but the organization we will leave behind. Certainly we are not superhuman; we do not know today what the crucial issues in behavior theory will be in 1975 and the nature of the organization which could best resolve them. But just as our own early efforts were influenced by the men who planned the Clinical Center and the methods for supporting its research operations and who then left before the programs got under way, so will our decisions determine the resources and affect the viability of the group we leave behind. We do not know at this time how to build such considerations into our planning, but it is a matter which merits our best thought and which may in the long run be every bit as important as any substantive contributions we are fortunate and ingenious enough to make.

ADDICTION RESEARCH CENTER

The work of the past year has in general continued the major lines of investigation with which the Center has been concerned for some time. Progress on several of these problems has been most encouraging, especially on development and testing of an effective narcotic antagonist.

Studies of the narcotic antagonist cyclazocine have taken an entirely different course during the past year. It had been shown previously that patients become tolerant to the dysphoric and sedative actions of this agent when it is administered chronically in large doses. During the last year it has been demonstrated that subjects do not become tolerant, however, to its narcotic antagonistic properties. Thus it has been possible to antagonize both the euphorogenic and physical dependence producing properties of morphine by administering cyclazocine orally in dose levels of 2 mg/70 kg twice daily. Subjects so treated who have received heroin in doses as large as 60 mg intravenously have experienced only a mild-to-moderate degree of euphoria. This dose would be normally highly toxic to nontolerant subjects. Overall experience indicates that this dose level of cyclazocine reduces the potency of narcotic analgesics by a factor of at least four to six times. In addition, subjects who are receiving cyclazocine chronically and who are at the same time receiving very large doses of morphine chronically (240 mg/day) do not develop a marked degree of physical dependence on morphine, and when abruptly withdrawn show a mild abstinence syndrome. It is proposed that cyclazocine be used in the ambulatory treatment of the abstinent addict, and it is felt that several gains may be realized by such a procedure:

1. Many of the deaths due to overdosage in nontolerant addicts on their first spree can be prevented by pretreatment with cyclazocine.

2. Abstinent patients who are driven to use of narcotics by adverse environmental circumstances will have a diminished drive to continue using narcotics compulsively, since the development of tolerance to and physical dependence on these agents will be inhibited and the euphorogenic actions of the agents blocked.

3. Further, it is felt that if either abstinence or drug-seeking behavior is conditioned, and conditioned abstinence as well as conditioned patterns of drug-seeking behavior are responsible for relapse, pretreatment with cyclazocine will insure the extinction of these conditioned responses since it will greatly decrease the chances that any reinforcement will occur.

Previously a very protracted abstinence syndrome has been demonstrated in the rat. Because the signs comprising this protracted syndrome were qualitatively different from the early signs of abstinence in the rat, they were collectively called secondary abstinence. Additional studies have indicated some rather surprising characteristics of this syndrome: (1) It appears to be an appetitive state, since both the intake of food and water in postaddict rats remains greater than food and water intake in comparable controls for many months. (2) There is evidence that the quantity of catecholamines excreted in the urine is decreased during the period of secondary abstinence. (3) Extensive study has indicated that protracted or secondary abstinence is probably one of the more powerful influences responsible for relapse in the rat. Thus it has shown that trained addict rats that have shown conditioned abstinence, as well as untrained addict rats that have not shown conditioned abstinence in the experimental chamber, relapse to almost an equal degree to the narcotic, etonitazene, when given access to this drug. These findings are felt to be important enough to attempt to again demonstrate protracted abstinence in man, using an experimental model which has not only theoretical significance but may be a tool for studying agents that can modify hyperirritability associated with protracted abstinence.

Studies of the alkaloids of *olioliuqui* (*Rivea Corymbosa*, morning glory seeds) indicate that they do cause marked perceptual distortions or hallucinations. It is, therefore, felt that the ingestion of morning glory seeds will not become widespread.

Isomers of tetrahydrocannabinol isolated and furnished through the courtesy of Prof. F. Korte of the University of Bonn, in Germany, are being studied in the hope that the active principle of marihuana might be established. Although the chemical structure of these iso-

meric mixtures are not known, it is quite apparent that all isomers of tetrahydrocannabinol do not have the same pharmacological action as marihuana, whereas others do.

Studies of alcoholics during parts of the addiction cycle, using the Addiction Research Center Inventory, indicate that the following symptoms are associated with alcohol withdrawal: low motivation, inefficiency, tension, anxiety, poor concentration, somatic complaints.

During the past year it has been shown that rats can be addicted to barbiturates, using the relatively inexpensive procedure of allowing them to obtain barbiturates in their drinking water. The withdrawal syndrome that results is characterized by an increase in body temperature, a reduction in food and water intake, partial or generalized convulsions, and, in some instances, death.

In addition, various factors influencing the rate of development of tolerance to electrical convulsions have been studied. Thus it has been shown that tolerance develops more rapidly in animals receiving convulsive stimuli than in animals receiving subconvulsive stimuli. It has been shown also that animals tolerant to electrically induced convulsions are cross tolerant to pentylenetetrazol induced convulsions.

The analysis of the personality characteristics of addict physicians as compared to normal physicians in practice is being continued. In the normal physician population, all scales fall within normal limits. In the physician addict population, all clinical scales were significantly abnormal except the hypomania and social introversion scales. The psychopathic deviate scale was most elevated, and the neurotic triad scales (hypochondriasis, depression and hysteria) were the next most abnormal parts of the profile.

In the study of conditioning in man, a very interesting and perhaps pertinent observation has been made: If subjects who have developed a conditioned response to an unconditioned stimulus (an electric shock) are told that there will be no more shocks, a conditioned response undergoes immediate and marked reduction in size.

Analysis of the data acquired during the Kentucky follow-up study continues. One of the

major findings of this study was that narcotic addiction was associated in this population with a loss of about one-third of the life expectancy. On the basis of history, it also appears that living subjects spent about one-third of their time addicted to narcotics, although at the time of interview less than 20 percent of the subjects living were using narcotics.

Studies have indicated that both nalorphine and morphine stimulate the incorporation of P³² into phosphatidyl inositol, phosphatidic acid, lysophosphatidyl inositol, phosphatidyl serine, phosphatidyl ethanolamine and diphosphoinositide. Morphine inhibits the incorporation of P³² into phosphatidyl choline.

Evidence has been obtained of muscarinic cholinergic neurones that facilitate the segmental pathways that mediate the flexor reflex.

I. Addictive Properties of New Analgesics and Pathophysiological Processes Associated with Physical Dependence

A. It has been shown that patients can be addicted to very large doses of nalorphine and become almost completely tolerant to the dysphoric and sedative actions of this drug. In addition, they are cross tolerant to the sedative and dysphoric effects of another narcotic antagonist, cyclazocine. When patients who are physically dependent on large quantities of nalorphine (240 mg/70 kg/day) are abruptly withdrawn, an abstinence syndrome emerges that is more closely related to the cyclazocine abstinence syndrome than to the morphine abstinence syndrome. Outstanding among its characteristics is the fact that the abstinence syndrome is not associated with compulsive drug-seeking behavior. It thus appears that the narcotic antagonists do produce a type of physical dependence that is qualitatively different from either the physical dependence produced by the narcotic analgesics or sedative-hypnotic agents.

B. Additional properties of the narcotic antagonist cyclazocine have been studied. It has been established that this agent is effective orally and that it has a very long duration of action. When modest doses of this drug are administered chronically in dose levels of 1 or 2 mg/70 kg orally twice daily, both the euphoro-

genic actions of morphine and heroin can be antagonized and the development of physical dependence on morphine can be markedly attenuated. Subjects receiving 2 mg/70 kg twice daily orally experience only very modest effects when morphine (60 to 120 mg) is administered subcutaneously or heroin (60 mg) is administered intravenously. These dose levels of cyclazocine allow the development of only a very low degree of physical dependence on morphine which results in most subjects in a very mild and barely clinically detectable abstinence syndrome. It is thought that this agent might be useful in the ambulatory treatment of the abstinent narcotic addict. It would certainly minimize the euphorogenic physical dependence producing properties of any drug that the patient might acquire while "on the street." In addition, chronic administration of this agent would almost certainly reduce the incidence, if not prevent deaths from overdosing in nontolerant addicts who again begin using drugs. Finally, it is thought that this might provide a tool for extinguishing any conditioned abstinence or drug-seeking behavior in addicts.

C. The study on the addiction liability of oxycodone has been completed. The overall conclusion is that oxycodone is approximately equipotent to morphine in producing subjective effects, as well as in suppressing abstinence in subjects physically dependent upon morphine.

D. Studies on the assessment of mixtures of fentanyl [N-(1-phenethyl - 4 - piperidinyl) propioanilide, ARC I-J-4] and droperidol [1-{1-[4-(p-fluorophenyl)-4-oxobutyl]-1,2,3,4-tetrahydro-4-pyridil}-2-benzimidazolinone, ARC VI-M-1] have been completed. Fentanyl in almost every respect is a typically morphine-like agent, and has been adjudged more euphorogenic than morphine. Droperidol produced subjective effects similar to those produced by barbiturates and the phenothiazine tranquilizers. Although, with regard to certain measures, the effects of droperidol were additive with those of fentanyl, in no instance was there any evidence that droperidol potentiated fentanyl. These studies indicated that, if anything, mixtures containing fentanyl plus droperidol were somewhat less euphorogenic than equi-effective doses of fentanyl alone.

E. Studies on the addiction liability of alpha d - 2 - acetoxy - 1, 2 - diphenyl - 3 - methyl - 4 -pyrrolidino-butane HC1 (Lilly 31518; ARC I-C-27) are nearly complete, and indicate this congener of *d*-propoxyphene is approximately one-third as potent as morphine in suppressing physical dependence. This value is commensurate with its analgesic action. At the present time studies are being conducted to quantitate the euphorogenic action of this compound.

F. Substitution studies on 6 - methylene - 6 - desoxy - 14 - hydroxy - dihydromorphine HC1 (Abbott A25443; ARC I-A-40) have been completed. This agent appears to be equipotent to morphine in suppressing abstinence in patients physically dependent on morphine.

G. Studies on the effects of barbiturates on post-rotatory nystagmus are being continued. A method for electrically recording eye movements has been adapted. Experiments are underway to currently determine if this method can provide an independent measure of the effects of sedative-hypnotic drugs for the purpose of calculating their relative potencies. In addition, experiments are underway to give additional insight into the mode of action of barbiturates in prolonging post-rotational nystagmus.

II. Acute and Chronic Intoxication with Drugs other than Analgesics and Barbiturates

A. *Alkaloids of olioliuqui (Rivea Corymbosa, morning glory seeds, ARC V-E-5).* In recent years concern has arisen about the ingestion of seeds of the common morning glory by students and maladapted persons of Bohemian habit. A number of years ago the effects of ingestion of ground, whole morning glory seeds were studied at the Addiction Research Center. Doses ranging up to 6 grams of the ground seeds caused only slight sedation and nausea in former morphine addicts. In 1960 Hofmann and Tscherter isolated *d*-lysergic acid amide, *d*-iso-lysergic acid amide, and chanoclavine from the seeds of *Rivea Corymbosa*. Dr. Hofmann made available to us an extract of the alkaloids of the seeds as well as a synthetic mixture of alkaloids simulating in composition those found in the seeds. In former addicts, doses as high as 5 mg of either the

mixture of the natural alkaloids or the mixture of the synthetic alkaloids caused predominantly sedative effects. Marked perceptual distortion did not occur and no hallucinations were reported. It is not felt that ingestion of the morning glory seeds will become widespread.

B. *Isomers of tetrahydrocannabinol.* Through the courtesy of Prof. F. Korte of the University of Bonn, in Germany, supplies of certain isomers of tetrahydrocannabinol, the active principle of marihuana, became available. Dr. Korte isolated tetrahydrocannabinol from hashish by extraction with methanol and column chromatography. The tetrahydrocannabinol so isolated was then separated by an 87-tube counter-current distribution procedure. Professor Korte could identify four isomers designated as 1, 2, 3 and 4. He succeeded in separating isomers 1 and 2 in pure form but could not separate isomers 3 and 4. Studies with these isomers indicate that isomer 1 is relatively inert, since no effects were observed even after doses of 2:5 mg/kg, whereas isomer 2 and a mixture of isomers 3 and 4 caused definite marihuana-like effects in a dose range of 120 to 360 mcg/kg. The original tetrahydrocannabinol (mixture of isomers 1, 2, 3 and 4) was also active in the same dose range, as was a mixture of isomers 2, 3 and 4, which suggests that the relative potencies of isomers 2, 3 and 4 are not too greatly different. The exact chemical structures of the various isomers have not yet been established.

It is hoped that these experiments will lead to the determination of the chemical structure of the active isomers and to the synthesis of a sufficient supply of active material to permit more detailed study of the pharmacology and psychopharmacology of marihuana that has been possible in the past. Since marihuana is one of the most widely used intoxicants in the world, and since the United States has a considerable problem of abuse of marihuana, the importance of such studies is evident.

III. Clinical Studies of Intoxication with Alcohol, Barbiturates and Related Drugs

Studies during the current year were chiefly focused on evaluating changes associated with

withdrawal of alcohol and the further validation of scales which are presumptive measures of differences between the behavior of alcoholics and narcotic addicts, on psychopathic deviation, and drug related behavior.

In the previous year, an MMPI scale was described which differentiated alcoholics and narcotic addicts (AAF). Within the opiate addict population, scores on this scale, on which alcoholics score high, are negatively correlated with time after withdrawal and a social maladaptation scale (SMF). In addition subjects eligible for research studies scored lower than addicts from the general hospital population. The AAF scale has been interpreted as a partial measure of inefficiency and denial of social maladaptation (opposite of obvious psychopathic behavior). To further validate these scales, scores were correlated with time after withdrawal of alcohol in alcoholics and recidivism in nonaddict criminals. The scores on the AAF scale were inversely related to time after withdrawal in alcoholics and the rate of recidivism in criminals. The SMF scale was positively correlated with rate of recidivism. In a somewhat similar analysis of the Personal Inventory and Inventory of Habits and Attitudes, the scales for showing obvious psychopathic deviation were consistently correlated with severity of addiction rather than with scales measuring acceptability for therapy, history of drug use by family or associates, history of excessive drug use, reasons for using drugs, or maladjustment.

Subjects being withdrawn from alcohol have been tested using the Addiction Research Center Inventory, a test that was designed to show drug effects. Preliminary analysis has shown that on certain scales there is almost no overlap in scores for subjects tested two days after withdrawal as compared with one week after withdrawal. Some symptoms associated with withdrawal of alcohol include low motivation, inefficiency, tension, anxiety, poor concentration and somatic complaints. Thus, the ARCI should prove to be a useful instrument for testing the utility of treatments that are designed to diminish withdrawal symptoms.

Elevation on the LSD-25 specific scale has been observed for the narcotic antagonists and during withdrawal from opiates and alcohol.

These findings suggest that antagonists of drugs which produce dependency as well as abstinence result in the production of psychotomimetic subjective effects.

Some differences between alcoholics and addicts on the Personal Inventory and Inventory of Habits and Attitudes are suggestive of possible points of emphasis in treatment. The alcoholic, as compared with the addict, finds more personal reasons for drug use. The difference, though rather small, suggests that the alcoholic would be more amenable to treatments which emphasize substitute gratification.

Further studies of personality of alcoholics and addicts are planned. An alcohol "withdrawal" scale will be developed. Correlates of scales which measure drinking habits will be explored. The Inventory of Habits and Attitudes will be administered to normal subjects to evaluate the significance of a number of scales. Differences between postalcoholics and postaddicts will be studied, using Cattell's 16 Personality Factor Test and Gough's California Psychological Inventory.

IV. Biochemistry of Addiction

Rats are being followed through a course of addiction to morphine, with special emphasis on the results of the infrequently reported phenomena observed during a period of prolonged secondary abstinence. The present experiment includes analyses of urine, brain, and adrenal glands for catecholamines, and observations on food and water intake, urine volume, body temperature and body weight. Addicted rats have now been followed through the fourth month of secondary abstinence. During addiction preliminary calculations show a significantly increased excretion of epinephrine, norepinephrine and dopamine; no changes in urine volume; increased body temperatures; decreased food and water intake, and decreased body weight for the first three weeks. During the first three or four days of abstinence the most notable changes were increased catecholamine excretion, the hyperthermia observed during addiction dropped to subnormal levels on the first day; after that, addict and control group body temperatures were equal. The decreased food and water intake of the first two

days had returned to normal values by the fourth day. Rough evaluation of the data of secondary abstinence from the second through the seventh week showed the same urine volumes for both control and addict groups; temperature was slightly but not significantly elevated in the addict group. The weight of the addict group steadily gained, almost reaching that of the control group by the seventh week. The addiction and primary withdrawal values of higher catecholamines excretion was reversed by the second month to values lower in the addict than in the control group.

This experiment was designed to show whether, during the period of secondary abstinence, alterations in oxidative and water metabolism in the rat are due to a slowly reversible disorder in hypothalamic-pituitary function, and whether this disorder may be related to an alteration in brain catecholamine metabolism. We hope to have a relatively complete picture of these relationships at the termination of the experiment.

Future experiments. A similar experiment using human subjects is planned.

V. Neurophysiology and Neuropharmacology of Chronic Intoxication with Barbiturates and Related Drugs

A. The Barbiturate Withdrawal Syndrome in Albino Rats. Wistar rats will drink increasing concentrations of sodium barbital in tap water if that is their only source of fluid. During the several months that the dose is increased, the rats are drowsy and their movements are incoordinated. Abrupt withdrawal of the drug results in an increase in body temperature, a reduction in food and water intake, partial or generalized convulsions and, in some cases, death.

B. Relative Roles of Subconvulsive and Convulsive Electrical Stimulation of the Cat Brain in the Development of Tolerance to Electroconvulsions. Progressively increasing intensities of electrical stimulation to the brain surface in cats finally results in a generalized convulsion. The daily repetition of this procedure results in a tolerance-like effect in that the current necessary to cause a convulsion gradually increases. A better understanding of this phenomenon

might have implications for tolerance mechanisms in drug addiction. The question arises as to whether the subconvulsive levels of electrical stimulation, the convulsion itself, or both, are important for the tolerance-like response to develop. During the past year, it has been demonstrated that subconvulsive electrical stimuli alone elevate the electroconvulsive threshold only a very small degree. However, the administration of electrical convulsions without preceding subconvulsive stimulation results in a much larger degree of threshold elevation. Thus the convulsion is the more important factor in the development of the tolerance-like response to repeated electroconvulsions.

C. Effect of Repeated Electroconvulsions on the Pentylenetetrazole (Metrazol) Convulsive Threshold. Another question that arises relative to the tolerance-like effect of repeated electroconvulsions is whether the phenomenon is restricted to electrically induced seizures, or if some cross tolerance develops to other convulsant agents. The pentylenetetrazole (Metrazol) convulsive threshold was determined in a group of cats prior to and following the repeated daily induction of electroconvulsions. It was found that the Metrazol convulsive threshold was significantly increased after a series of electroconvulsions had induced the usual electroconvulsive tolerance effect so that a cross tolerance was demonstrated.

It is planned to determine whether the daily repetition of Metrazol induced convulsions will result in an increase of the threshold for electroconvulsions. The effect of the daily administrations of sodium barbital on the tolerance-like response to electroconvulsions will also be studied.

VI. Psychological Studies of Addiction

The objectives of these projects are much the same as indicated previously, alterations have been made chiefly in techniques and methods of analysis. The studies were designed (1) to define more clearly personality characteristics of the narcotic addict and show how these differ from "normal" personalities, (2) to develop more refined techniques for measuring subjective effects of analgesic, tranquilizing, analeptic, and psychotomimetic drugs, (3)

to relate differential measures of drug effects to specific personality characteristics, (4) to investigate personality variables which may control behavior in the psychopath and relate these to the misuse of opiates and alcohol, and (5) to study drug-produced changes in conditioned and unconditioned responses in animals.

Investigations with the Addiction Research Center Inventory (ARCI) were extended to several nonaddict populations which included hospitalized psychiatric, normal, criminal, and alcoholic subjects. Various samplings are also being tested independently by other investigators using the ARCI with and without drug administration. The general purpose of investigations using nonopiate addicts as well as non-addict subjects is to determine the degree of similarity between personality types, psychopathology and drug effects, and differences between opiate addicts and other clinical groups, as well as some correlates of changes in subjective experience.

Opiate addicts under no-drug conditions can be effectively differentiated from psychiatric subjects using ARCI scales. The greatest differentiation is on the empirical drug scales, suggesting that the general subjective changes produced by drugs are similar in some respects to those associated with mental illness. Several other types of scales also differentiate psychiatric patients from opiate addicts. Thus the former show more neurotic characteristics than do opiate addicts, and greater feeling of inadequacy, uncriticality and less sentimentality, whereas addicts show more psychopathic response. The most surprising finding is the high degree of euphoria in the psychiatric group as compared with addicts, except for patients who have been diagnosed as depressives. Within the mentally ill group, ARCI scales are more highly correlated with the drug administered, MMPI scales however are more highly correlated with psychiatric diagnoses.

Results on criminals and normal subjects are not fully analyzed as yet. These data will permit an evaluation of some of the similarities of the symptomatology of mental illness and drug induced changes and other determinants of behavior (including current physical health, special kinds of stress, habits referring to smoking, use of drugs, etc.) which might affect

subjective report. Acute change in general as opposed to a chronic condition appears to be more highly correlated with report of change in subjective experience. Comparison of opiate addicts under no-drug, placebo, morphine, pentobarbital, cyclazocine, and nalorphine on the ARCI was focused primarily on the differences of morphine and the latter two drugs since these are antagonists of morphine. Prior studies indicated that all psychologically active drugs have some effects in common (non-specific effects) which are reflected in a "general drug-effect scale" (GDE), and a scale of "Reactivity" and all drugs in the present series produced significant elevations on GDE. Morphine was quite different from cyclazocine in a number of respects. Morphine produced more euphoria, efficiency is markedly impaired by cyclazocine and nalorphine, and some psychotomimetic effects are induced by these drugs, but not by morphine or pentobarbital. The effects of nalorphine and cyclazocine appear to be intermediate between the patterns of effect associated with pentobarbital and LSD-25.

One general objective of research with opiate addicts is concerned with the isolation of characteristics that are associated with phases of the cycle of addiction. Currently such phases are being studied with the ARCI and the Lexington Personality Inventory (LPI).

Physician addicts were compared further with normal practicing physicians (collected by Drs. W. G. Dahlstrom and R. S. Spain) and with addicts of the general hospital population. In the practicing physician, all scales fell within normal limits. In sharp contrast, the physician addict profile showed an elevation on all clinical scales except Hypomania and Social Introversion. Although the Psychopathic Deviate was the most elevated of all scales in the physician addict group, the mean score was significantly lower than that for the "general" hospitalized addict patients. The physician addict also showed a marked elevation in the "neurotic triad" scales (Hypochondriasis, Depression and Hysteria). Various factor analysis produced strong confirming evidence that the physician addict is significantly distinguished from the normal physician by factors of psychopathy, neuroticism, depression and social maladaptation. These findings support the hypoth-

esis that personality disturbance is an important factor in the addiction process.

Several further studies on conditioned avoidance behavior under both drug and no-drug conditions showed an increase in spontaneous activity (crossing from one side of a shuttle box to the other) under morphine. This is in contrast to the usual depressive effects of this drug, especially on appetitively controlled responses. Somewhat similar results were found for amphetamine, but not for pentobarbital. Studies on the antecedents of this effect in addition to those reported previously are being continued. It appears that in situations in which animals have not acquired aversive behavior, morphine tends to depress action; whereas, under conditions which have been noxious, morphine and perhaps other analgesics tend to activate behavior. These "release" or disinhibition effects are being studied because they may have some relation to the addiction liability of opiates in man.

VII. The Mode of Action of Central Nervous System Depressants

It has been previously demonstrated that dogs infused with morphine sulfate for 7 to 8 hours exhibit an acute abstinence syndrome when nalorphine is administered. Various investigators have felt that perhaps the syndrome thus precipitated was not indeed an abstinence syndrome but was a consequence of the unopposed excitatory actions of morphine resulting from the selective blockade of the depressant effects of morphine by nalorphine. To determine if the acute abstinence syndrome was indeed the consequence of the predominance of the excitatory actions of morphine, the following experiment was done: Dogs were given 20 mg of nalorphine and then, at a time when actions of nalorphine were nearly maximal, 20 mg of morphine was administered. Following the administration of morphine, a syndrome did emerge that superficially resembled the acute abstinence syndrome seen in both the acutely and chronically physically dependent dogs. Close analysis and comparison of signs, however, indicated that this syndrome was quite different from the acute abstinence syndrome.

A continuing effort is being made to investigate the role of neurohumors in the mediation and modulation of the flexor reflex of the chronic spinal dog. It has been shown that this reflex is enhanced both by directly and indirectly acting sympathomimetic amines and that these actions are antagonized by an alpha adrenergic blocker such as dibenzyline, but not by beta blocker such as DCI. Recent studies have indicated that whereas neither methylatropine nor atropine affect the flexor reflex when given alone, eserine will increase the amplitude of this reflex, and this increase in amplitude is antagonized by atropine, but not by methylatropine. Since atropine does not suppress the flexor reflex *per se*, it is felt that the muscarinic cholinergic neurones cannot be in the segmental pathway mediating this reflex, but rather modulate this pathway.

It has been tentatively concluded that in the spinal cord, below the level of transection, there are both adrenergic and cholinergic facilitatory modulating pathways that have a low level of spontaneous activity. This finding provides additional support for the concept that there is pharmacological redundancy in the spinal cord.

Studies have also been initiated to investigate the kinetics of the nalorphine-morphine interaction on the spinal cord reflexes of the chronic spinal dog. Preliminary studies indicate that although nalorphine is approximately equipotent to morphine in suppressing the spinal cord flexor reflex over a limited dose range, the dose response curve of this agent has a gentler slope than the morphine dose response curve, and also shows a clear-cut plateau indicating that nalorphine has a maximal activity that is considerably less than that of morphine.

VIII. Conditioning Factors in Opiate Addiction and Habituation (relapse)

During fiscal year 1965, further studies on the conditionability of "wet dogs" (a morphine-abstinence sign in the rat) were conducted, and an attempt was made to isolate the roles of this variable, of reinforcement of opioid-acquisitory behavior by suppression of morphine-abstinence phenomena, and of previous physical dependence *per se* in the genesis

of "relapse" in the rat. The experimental design was basically the same as that described in the Annual Report for January 1963-June 1964, with the following modifications:

A. Anise-flavor was eliminated altogether and replaced as a discriminative cue by a set of tactile and visual cues, identifying either the etonitazene tube or the water tube for alternate rats in the two "trained" groups (AET, morphine-addicted, etonitazene-trained, and SET, saline-injected, etonitazene-trained), a swell as in the "relapse" tests for all four groups.

B. The two "non-trained" groups, (AUT, morphine-addicted, untrained, and SUT, saline-injected, untrained) remained in their individual home cages throughout the six-week "training period" (for AET and SET), though they received morphine (AUT) or saline (SUT) injections on the same schedule as their corresponding "trained" groups. Thus, AUT and SUT had no experience in the linear mazes until the first "relapse" test.

Because of a series of unfortunate circumstances (two epizootics of murine virus pneumonitis and one of staphylococcus peritonitis, probably induced by an accidental break in sterile injection technique), the study had to be repeated three times in order to obtain sufficient numbers of rats in the four groups for evaluation. To-date, sufficient data for evaluation have been collected for the first three "relapse" tests.

Although statistical analyses of these date have not yet been completed, the gross trends are quite clear and may be considered in relation to the two major dependent variables.

1. Unconditioned and conditioned "wet dogs" in "relapse" tests. As predicted by the theory, "wet dog" counts were higher in the linear maze than in the home cage for AET and higher in the home cage than in the linear maze for AUT. In other words, both groups of "postaddict" rats displayed higher "wet dog" counts in their respective "places of abstinence" each day during the six-week training period preceding the first "relapse" test. These findings confirm the conclusion reached in the original study, that "physical dependence" can be conditioned in the classical manner.

2. "Free choice" drinking of ETZ⁵ and H₂O in "relapse" tests. It will be recalled that in the original study both "postaddict" groups (then designated as ET and ENT) consumed significantly more fluid in the form of ETZ⁵ than either control group (then designated at CT and CNT), but in this respect ET and ENT did not differ on the first "relapse" test, and ENT surpassed ET on the next four "relapse" tests. This unexpected finding was tentatively attributed to the use of anise flavor as a discriminative cue, on the assumption that the flavor might have set a "ceiling" on the amounts of ETZ⁵ that any rat would consume. The results of the present study however do not support that assumption. Indeed, in terms of the relative amounts of ETZ⁵ consumed by the various groups, the present data are very similar to those obtained in the original study. Hence it must be concluded that prior physical dependence on morphine is, *per se*, the principal factor responsible for the greater "free choice" consumption of ETZ⁵ by "postaddict" than by control rats regardless of prior classical or instrumental conditioning.

Another similarity in the results obtained in the original and present studies is the evidence that for nonaddicted, control rats ETZ⁵ is aversive. Thus, in successive relapse tests, the percent of ETZ⁵ consumed by SET and SUT fell progressively, that for SET (previous experience with ETZ⁵) being manifested on the first "relapse" test in comparison with SUT (no previous experience with ETZ⁵). The fall in percent of ETZ⁵ consumed on the third "relapse" test (44 days abstinent) by AET and AUT may also be indicative of the aversiveness of ETZ⁵, emerging as the "need" for etonitazene (whatever its physiological basis may be) declined.

From the data obtained in both the original and the present studies, it is now clear that in the rat, previous physical dependence on morphine is *per se* an important factor in generating a disposition to "relapse" long after morphine withdrawal. For the reasons mentioned in the Annual Report for January 1963-June 1964, it is unlikely that such a disposition is due to residual tolerance to opioids. Rather, the non-aversiveness of etonitazene for "postad-

dict" rats (relative to nonaddict rats) may be due to persistence of homeostatic imbalance as manifested in the long-enduring "secondary" abstinence syndrome, of which total "wet dog" counts (unconditioned plus conditioned) may be an indication in the present study. These findings and conclusions emphasize the need for intensive research on the possibility that "secondary abstinence" also occurs in man.

Confirmation of the conditionability (classical) of at least one morphine-abstinence phenomenon ("wet dogs") in the rat supports the hypothesis that in man relapse may be due, in part, to the recurrence of abstinence phenomena (as conditioned responses) long after morphine-withdrawal. Theoretically, prior reinforcement of opioid-acquisitory behavior by suppression of morphine-abstinence phenomena during periods of active addiction to morphine should also play a role in generating a disposition to relapse, but the influence of this factor as well as of conditioning of "wet dogs" on etonitazene-drinking in "relapse" tests could not be demonstrated by the techniques employed in both the original and the present studies in the rat. Probably a more differentiating technique than "free choice" drinking of ETZ⁵ or H₂O over a 12-hour period will have to be devised to isolate the roles of these two kinds of conditioning factors in relapse.

During the coming year attention will be focused on the hypothetical "effort" variable in generating a disposition to relapse. Only morphine-addicted rats will be used, maintained on one intraperitoneal injection of morphine (200 mg/kg) at 8 a.m. each morning. For one group, ETZ¹⁰ will be available for drinking without other "effort" throughout the nocturnal abstinence periods on alternate days. For another group, ETZ¹⁰ will also be available during the same period, but the animals will have to "work" for the drug, i.e., press a lever to gain access to the drinking tube every 10 minutes or so (exact schedule to be determined empirically). Amounts of ETZ¹⁰ consumed by the two groups during a six-week "training" period will be equalized. After completion of "training," all injections will be terminated and subsequently "relapse" tests will be conducted (tentatively by the "free choice" tech-

nique) with H₂O and ETZ⁵ freely available without "effort" on both groups. It is postulated that the "hustlers" (rate trained to "work" for ETZ¹⁰) will consume more ETZ⁵ than the "non-hustlers."

IX. Experimental Studies with Human Subjects

In the past year we have continued the series of studies of several years' duration on the effects of morphine upon responses of the autonomic nervous system to conditioned and unconditioned stimuli.

Interest in the effects of morphine upon autonomic responses stems from a preliminary hypothesis that such effects might play a role in the production by morphine of analgesia as well as being related to the addiction liability of morphine. This hypothesis was suggested on the basis of evidence from other sources that (1) morphine reduced anxiety in clinical use, and (2) that the analgesia produced by morphine may reflect a change in reaction to stimuli rather than a change in perceptual threshold for stimuli.

The autonomic response upon which we have focused thus far has been the electrodermal response. A recurrent observation has been that morphine attenuates the increase in basal skin conductance which normally occurs when noxious stimuli are applied to the subject. On the basis of the reduced tonic level of autonomic activity, one might well expect that reflexive or phasic activity would be correspondingly reduced. In regard to responses to unconditioned stimuli, however, we have found that the subjects continue to show normal phasic responsibility to unconditioned stimuli after morphine injection.

In regard to the acquisition of conditioned electrodermal phasic responses, there was a moderate reduction found. Such a reduction is in keeping with what might be expected from the reduction by morphine of tonic levels of activity, and perhaps reduction of other facilitating sets. The latter conditions, however, would also lead to the prediction that unconditioned, evoked responses would likewise be reduced, which was not observed. Alternatively, if one assumed that the reduction in basal skin conductance was only a measure of the reduced

significance for the subject of the unconditioned shock stimulus, perhaps in terms of anticipatory anxiety, and that morphine did not alter the perception of actual stimuli, one could then predict the obtained differential effect of morphine upon conditioned versus unconditioned responses.

Within such a conceptual framework, the prediction could be made that the greater the reduction of the significance of the unconditioned stimulus, the greater would be the reduction of the response conditioned to that stimulus. In the last annual report an observation was mentioned which bears on this line of thought, and during the current year an additional pertinent observation has been made. The more recent observation was that if, after a differential conditioned electrodermal response has been established to tones with electric shock as the unconditioned stimulus, the subject is told that there will be no more shock, the conditioned response undergoes immediate and marked reduction in size. It is as if there were no appreciable strength of connection between the conditioned stimuli and the conditioned response. These observations would support a cognition rather than a reinforcement type theory of learning, since even though a reduction of significance of the unconditioned stimulus by morphine could be considered as a reduction in level of drive in a reinforcement theory, such drive reduction would be expected to reduce the unconditioned as well as the conditioned response.

These observations are of interest in regard to conditioning phenomena and learning theory, and during the coming year we will plan some experiments along this line of interest in addition to continuing our experiments on the effects of morphine and other drugs on conditioned responses.

X. Social Science Section

Analysis of the data of the Kentucky follow-up study continued through the year and reports on part of the data were prepared for publication. Major findings included a high death rate, with a loss of about one-third of life expectancy at time of first admission for the male subjects. The loss of life expectancy was

somewhat less for the women. Most of the living subjects were not addicted to narcotics, though many of the men had shifted to barbiturates or alcohol. Of the thousands of man-years between first admission and death or the time when living subjects were located and interviewed, about one-third were spent addicted to narcotics.

Data collection was completed on the Puerto Rico follow-up study early in the year. Editing, coding, and analysis of data is in progress.

One of the purposes of these two studies was to develop and validate a methodology to see if addicts who had been hospitalized up to 25 years earlier could be located, if they would cooperate with the field interviewers, and if objective information could be obtained from them and other sources to permit reliable classification of subjects with regard to addiction status. All of these questions were answered in the affirmative. In the Kentucky study a death certificate or an interview was obtained for all but 3 of 266 subjects. In Puerto Rico no effort was made to locate those who had left that commonwealth, so only 109 subjects were located and interviewed, but some posthospital information was secured for 97 percent of the 243 subjects. If given a high quality of field personnel, almost all subjects can be located if the purposes of the study justify the needed expenditure of time and money.

Once located, the subjects were surprisingly cooperative. Only 3 of 112 subjects in Puerto Rico, and none of the 119 located in the Kentucky study, refused to be interviewed. Almost all who were interviewed, even those who were using narcotics regularly, were willing to give a urine specimen to be tested for drugs. Data were also available from relatives, physicians, and official records, so that confirmation could be obtained for most of the abstinence claimed.

During the year the IBM file of all admissions to the Fort Worth and Lexington hospitals was brought up-to-date. This file will furnish data for detailed analysis of admissions and readmissions over the next few years. Initial examination of data on male patients admitted to the Lexington hospital shows that from 37 to 51 percent of those first admitted in any year from 1935 to 1949 had one or more

later readmissions. Readmission rates were higher for voluntary patients than for prisoners, and for whites than for non-whites.

The IBM file was used to identify those addict patients who were hospitalized at Lexington on one date in 1962, and the geographical mobility of this sample was studied. It was found that they were not more mobile from birth to the onset of addiction than the U. S. population, and that they did not lead a transient way of life after their initial hospitalization. Despite this general absence of association between addiction and mobility, distinct patterns of population movement were found to characterize the several ethnic groups—white, Puerto Rican and Negro. Migration and inter-generation mobility were most common among the Puerto Rican and Negro addicts, while the native white addicts were more stable in their place of residence. These and other ethnic mobility differences, however, were consistent with those of their respective base populations in the United States.

One project, initiated late in the year, will investigate the employment history of samples of current Lexington admissions and relate this to drug history. In addition to the detailed information expected from these interviews, they will furnish a criterion against which the reliability of certain items of information routinely obtained by the hospital at the time of a patient's admission can be compared.

The section has furnished consultation to the Kentucky State Department of Health on an analysis of data already collected, which may furnish some estimate of the prevalence of addiction to narcotics and barbiturates which is maintained on legally acquired drugs. This might develop into a field study by the Department of Health to ascertain the prevalence of such addiction in the state.

XI. Chemical Pharmacology

Nalorphine causes a reduction in the central nervous system levels of N-C¹⁴-methyl labeled morphine in tolerant dogs (42 to 56 percent) at a time 65 minutes after administration of labeled morphine, but no statistical change at either 165 or 275 minutes. A lowering of the level of drug (5 to 75 percent) was also ob-

served in the heart, lung, liver and kidney following nalorphine. Plasma levels of free drug in the antagonized dogs were essentially lower than control, whereas conjugated levels of drug were higher than control values at certain time intervals. Chromatographic studies provided no evidence for the existence of N-C¹⁴-methyl labeled metabolite of morphine.

The recovery of free H³-cyclazocine in urine as a percent of the injected dose was 4.4 percent, and as conjugated drug was 33.3 percent. From the feces, 4.3 and 1.6 percent was recovered as free and conjugated drug, respectively. A total of 43.7 percent of the injected dose was accounted for. Levels of labeled drug were 1300 to 2500 ng/gm in the kidney, spleen, and liver after one hour. Cerebral cortical gray matter and white matter concentrations were about 1200 ng/gm and 800 ng/gm, respectively, at one hour.

Morphine and nalorphine stimulated the incorporation of ³²P_i into phosphatidyl inositol (PI), phosphatidic acid (PA), lysophosphatidyl inositol (LPI), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), and diphosphoinositide (DPI) in cerebral cortex slices after a two hour incubation at 37° C. The effect of morphine on the incorporation of glycerol-1,3-C¹⁴ and inositol-C¹⁴ into phospholipids was similar to that observed for ³²P_i. Morphine inhibited the incorporation of ³²P_i into phosphatidyl choline (PC).

LABORATORY OF SOCIO-ENVIRONMENTAL STUDIES

The general orientation of the work continues to be toward the study of the effects of social experience on personality and behavior. Subsumed by this generic interest are several substantive areas of concentration. The first is that of family and personality development, in which there has been an increasing amount of work in recent years. A second area deals with clinical settings and the social characteristics of their patients. The third area, dealing with occupational life and its concomitants, has become a major focus of interest in the Laboratory. Finally, the methodology of the social sciences stands as a fourth distinct area of concentration, for it is now receiving considerable

attention. Some of the methodological inquiries, it will be seen, are directly linked to the investigation of substantive problems while others are independent of particular content interests. In describing the work of the Laboratory, special note must also be made of the source of data of several of the studies. As a result of opportunities for overseas assignments, a number of investigations have been conducted in foreign lands. A few of these are replications of studies that have also been done in the United States, thus giving a distinctive crosscultural flavor to part of the body of the ongoing work.

We turn now to a more detailed discussion of the studies within each of the four areas enumerated above.

Family and Personality Development

This continues to be the area of heaviest concentration. Almost all the Laboratory's investigators currently devote at least part of their research effort to problems in this domain. Yet there is considerable diversity in the specific issues under investigation, the samples taken for study, and the methods employed in the research.

One issue, that of moral development, is the major research concern of Dr. Roger Burton. Pursuing the program he initiated several years ago, he has, in the past year, examined the relationship between the behavior of nursery school children in a temptation situation and their fantasies evoked during doll play. He aimed to find: (1) if it is possible to predict from expressed themes during doll play whether a child will cheat or conform to the rules of a game, and, (2) if, following cheating in the game, expressions of guilt are voiced by children in subsequent doll play. The importance of this work lies, in part, in the assumption that feelings of guilt or remorse are controlling mechanisms that contribute to the learning of and adherence to norms and rules. Although it was found that the fantasies had no predictive value for cheating, there is an interesting difference in the changes which occur after temptation in the fantasies of the cheaters and non-cheaters. The difference is not in the direct expression of guilt, however.

It is, rather, that the cheaters more often emphasize parental nurturance and protectiveness while the fantasies of the conformers stress parental punitiveness. This, in turn, provides a cue as to relevant familial antecedents of moral development in children.

Dr. Phyllis Scott has also utilized the experimental method in a provocative investigation of the effects of adult reinforcement on child behavior. In this case, however, the experiment was not in a laboratory but was imposed on the naturalistic setting of a nursery school. Specifically, contemporary learning theory was brought to bear on the experimental modification of the social behavior of a 4-year-old child who was having difficulty in relations with both peers and teachers. An experimenter systematically applied positive reinforcements following acceptable social behavior and ignored unacceptable behavior. The over-all result of the experiment was an increase in the frequency of approved and a decrease in the frequency of disapproved acts. There was a high degree of agreement between two sets of behavioral observations made, one by observers uninformed as to the hypothesis being tested, the other by an informed observer. Data were collected showing how reinforcement affected not only the child to whom it was directly applied, but also his peers. Modification of the subject's behavior probably resulted both from the actions of the experimenter and from schoolmates. Thus, one of the gains resulting from work in the naturalistic setting is that it makes it possible to assess the effects of the experimental manipulation on the social context of the subject. In interpreting the experimental results, Dr. Scott introduces a note of caution. She points out that from this experiment alone, it is impossible to tell how much of the positive effect on the child's behavior came about from the reinforcing properties of the adult or from the fact that her presence also inhibited negative behavior.

The work of Dr. John Campbell focuses on cognitive and conceptual development in children and currently he is working on two studies dealing with these issues. One study concerns the definitions and perceptions of illness among hospitalized children of ages six through twelve. In addition to their definitions

of illness, it has been possible to get information on their conceptions of prevention and treatment of illness and the "proper" behavior of children during illness. Many of the questions addressed to the children are also asked of their parents, thus permitting parent-child comparisons to be made. The study will be more prominently described in subsequent reports, as the data are still in the process of being collected. The second study, begun while Dr. Campbell was in England, deals with children's conceptions of nationality and national differences and their emotional reactions to these perceived differences. Several hundred children from the ages of six to twelve were presented with a series of photographs and, although all of the pictures were of Englishmen, they were asked to judge whether those portrayed in the photographs were English or of other nationalities. They were also asked to indicate the extent of their liking or dislike for the photographed individuals. Preliminary findings indicate that the English children voice much more positive feelings for people they judge to be English; this association, however, is significantly more pronounced among the younger children than the older. Another aspect of the study deals with the conceptions of geography and spatial distributions of different nationalities. This work, too, will be reported more fully as the analyses progress.

For several years, Dr. Morris Rosenberg has concerned himself with self-image among adolescents, its social antecedents and consequences. This work has resulted in a notable book (awarded an American Association for Advancement of Science prize) recently published by the Princeton University Press, entitled *Society and the Adolescent Self-Image*. Dr. Rosenberg has observed that there are a number of critical aspects of self-image that have not yet been investigated. One of these deals with the relationship between one's image of himself and the way he appears in the eyes of his friends and acquaintances. While in England during 1963-64 Rosenberg conducted a study among adolescent British school children directed at the problem of self-and-other-images. These data have only recently been prepared for analysis and there are no results

to report at this time. However, the work represents a step toward learning more about the relationships between a crucial personality variable and interpersonal interactions. It can also be noted that some of the material collected in England corresponds to data collected earlier among American adolescents. Thus, this work will deal both with the problem of self-other perceptions and cross-national aspects of adolescent self-image.

The data of Dr. William Caudill in the area of child development are also cross-national. As part of his research in Japan, Caudill has made very detailed home observations of the behavior of thirty 3-to-4-month old infants and their mothers. Simultaneously, data were gathered among thirty matched infant-mother pairs in the United States, using the same observational protocols. The families in both samples are middle class. The early findings from this study reveal some interesting and exciting cross-national similarities and differences. Contrary to "common sense" expectations, the differences are greater for the behavior of the infants in the two cultures than for the behavior of the mothers. American infants are more active and more vocal than their Japanese counterparts, and are more involved in the manipulation of physical objects in their environment. American mothers leave their infants alone more frequently, but there is more vocal communication from American mothers to their infants. Japanese mothers, on the other hand, are in closer physical proximity to their infants and seem to be communicating more in non-verbal ways—such as holding and rocking the infant. Within the Japanese sample, there are differences in these mother-infant behaviors that are associated with the occupational styles of the families. Thus, in families where the husband is engaged in a salaried white collar occupation, behavior is more in the direction of that found in the United States. Where the father is a shopkeeper or engaged in some other independent calling, the observed behavior is more characteristically Japanese. Even at this early stage of the data analysis, therefore, it is possible to see how soon after birth a culture begins to influence the behavior of its members. Cross-national

comparisons, such as Caudill is making, enable us to discern these effects in a way that would otherwise be most difficult. A follow-up study of cases in both cultures is now being carried out.

The next two studies were conducted in Turin, Italy, a large industrialized city. During 1962-63, Dr. Leonard Pearlin conducted a study in this city which closely followed earlier work done by Dr. Melvin Kohn on social class and childrearing values in an American city. The Italian middle class has been found to emphasize self-control, self-direction, and autonomy in their children, while working class individuals give priority to obedience and conformity to rules and conventions of behavior. These same class patterns were among the central findings of Kohn's study. Through examination of the Italian data it has been possible to locate some of the social sources of these striking class differences. One such source appears to be in the relations with the larger family system. Both middle and working class Italians are closely attached to their families, as evidenced by frequency of visits. There seems, however, to be a class difference in the quality of the attachment. Thus, working class individuals, more than those from the middle class, are likely to report an identity of views and ideas with their relatives. In other words, there is more intra-family consensus in the working class. This suggests, in turn, that the working class valuation of conformity is part of a more general intergenerational continuity in transmitting values and in ways of dealing with the world. Certain occupational experiences of fathers are also relevant to the emphasis given conformity or autonomy. For example, when one works in a situation allowing considerable self-direction and autonomy, then he will come to value these as characteristics in his child; if, however, he has little opportunity for decision making, if his work is closely regulated by others, then he is more likely to stress conformity as a desired characteristic. In addition to the broad cross-national comparisons afforded by this study, it also provides an opportunity to demonstrate some of the social structural routes of childrearing values.

The data from Dr. Pearlin's Italian study come from structured interviews with over 850

Turinese. From this group of respondents, Dr. Marian Yarrow selected a subsample of 72 mother-father pairs and ten-year-old children to study social class and parental differences in parent-child interactions. Experimental situations involving problem solving were used. The child was observed in the home on two occasions. In one session the experiments were conducted with child and father; in another, with child and mother. Behavioral aspects studied include such dimensions as parent and child involvement in task achievement, cooperation, disagreement, and child's adherence to rules of the tasks. In addition to these observations, interviews were conducted with the child concerning his achievement values and aspirational levels. In this study it is possible to consider together children's values, parent-child behavior under controlled conditions and the parental values obtained from Dr. Pearlin's study. Dr. Harry Scarr has joined Drs. Yarrow and Pearlin in analyzing these data. In analyses completed thus far, a separateness of value and behavioral domains appears. The very considerable social class variation in parental values is not paralleled by behavioral differences, either in child behavior or in parent-child interaction in the experimental situations.

Clinical Settings and their Patients

The Laboratory has a long-standing history of studies in the structure of mental institutions, treatment processes and psychiatric patients. This interest is currently being pursued through the programs of Drs. William Caudill and Carmi Schooler, working in Japan and the United States, respectively. Much of this work is independent, but at some points their interests converge into very revealing cross-national comparisons.

Schooler's studies are made up of a series of related experimental investigations dealing with a group that tends to fall into obscurity in large mental hospitals; namely, chronic schizophrenics. One of his central aims in studying this group has been to observe what he considers their primary deficit: the inability to function in interpersonal relations or otherwise meet the demands of a social situation. Thus, in one experiment begun in 1964, he at-

tempted to assess the effects of social rewards on ability to master simple learning tasks. Analyses of the data thus far indicate that social praise, apparently as a result of anxiety aroused by this kind of reinforcement, tends to hinder rather than enhance ability to learn novel tasks. Schooler's interests in the effects of social conditions on the intellectual and social functioning of chronic schizophrenics has been extended in the past year to include observations of autonomic physiological functions. This work has been done in collaboration with Dr. Theodore Zahn of the Laboratory of Psychology. The main feature of their experiment was the construction of situations requiring different degrees of social cooperation with others in order to perform problem-solving tasks. In their analysis, the experimenters will then be able to see how variations in closeness of interrelatedness with others affect both patients' performance in dealing with the task and their autonomic functioning.

Dr. William Caudill, in earlier work with a sample of approximately 800 patients representing all admissions in 1958 to four representative psychiatric hospitals in Tokyo, has shown an overrepresentation of eldest sons and youngest daughters among schizophrenic patients; this is in contrast to Schooler's previous findings for American schizophrenics where youngest daughters are overrepresented, but there is not general finding for sons.

Using the 1958 Japanese sample, Schooler and Caudill have reported earlier on differences in symptom patterns for Japanese and American schizophrenics. Currently, a factor analysis has been made of all symptoms occurring in at least 10 percent of the 800 Japanese cases. For both male and female patients, a four-factor structure quite cleanly accounts for the variation in the data. These four factors may be labelled: 1) behavior disorder, 2) depression, 3) schizophrenia, and 4) *shinkeishitsusho*. The last is a distinctly Japanese neurotic syndrome made up of phobias, bodily complaints, and tense interpersonal relations. Of special interest is the fact that one of the hospitals specializes in patients with *shinkeishitsusho*, whereas the other three hospitals do not do so and also do not use this diagnostic term. Nevertheless, when a factor analysis is done on

the symptoms of patients at these latter three hospitals, a very clear *shinkeishitsusho* factor still emerges. Thus, this neurotic syndrome seems particularly Japanese. The next step in the analysis is to examine the distribution of the four factors by such variables as sibling position, social class, and occupational style of life.

The analysis by Caudill and Schooler of the Japanese data from 1958 is preparatory to a similar analysis which will be carried out on a second sample of approximately 1,000 cases representing all admissions to five psychiatric hospitals in Tokyo collected last year by Caudill while he was working in Japan. It is also planned to collect and analyze data from American hospitals for comparative purposes, and consideration is being given to the idea of adding a sample of Taiwanese patients in order to gain the perspective afforded by the addition of a third culture.

Social-Psychological Correlates of Occupation

As stated at the outset, interest in occupational life and its social and psychological consequences are of more prominent interest in the Laboratory than in previous years. The Laboratory has devoted a considerable share of its resources to this area.

The largest research effort is represented by the study being conducted by Drs. Kohn, Schooler, and Rosenberg. The goals and purposes of this investigation were presented in last year's report and will not be repeated here. During the past year, 3,000 interviews with a representative cross-section of the labor force of the Nation were successfully completed. The coding and preparation of these interviews for computer processing is now at the halfway mark, and it is anticipated that it will be possible to begin the analysis of these data at the end of the year. This is a massive undertaking, but will provide more comprehensive information about occupational life and its concomitants than heretofore known about this very important area.

Dr. Roger Burton is conducting an inquiry into professional musicians—an occupation that he has known from first-hand experience. In 1955, as part of his research in another in-

stitution, Burton collected a considerable body of psychological test material from a group of West Coast musicians. Since that time, they have undergone a good deal of occupational stress, largely resulting from the termination of contracts with motion picture studios. In 1962, Burton repeated his earlier work with many of the same individuals who participated in the earlier study with the purpose of finding some of the correlates of successful coping with the difficult transitional period that the occupational group experienced. Comparing the successful copers with those who left the field through attrition, he finds among the former more emotional stability, ascendancy, and masculinity, and less introversion. One of the surprising results was that when the test scores of the successful group are compared at the two periods in time, it appears that some psychological growth took place as a result of the demanding professional experiences through which they passed. This is inferred from the fact that on several of the tests these men scored more positively in 1962 than in 1955. In general, this study nicely illustrates that the effects of occupational experiences result from an interplay between the occupational demands imposed upon individuals and the psychological characteristics the individuals bring to the occupation.

Methodology of the Social Sciences

There is a constant need in studies of human behavior to challenge the adequacy of the data with which we work and to seek improved analytical procedures. Indeed, it is sometimes contended that the conceptualizations of problems in the behavioral sciences is currently more sophisticated than are methods to investigate them. Currently in the Laboratory, there are several efforts directed at the determination of the kinds of data most suited to answer validly and reliably certain research questions, and to finding analytical procedures and techniques which can bring maximum yield from the data. As noted earlier, some of this work is directly linked to substantive issues, while other is independent of substantive problems. Similarly, in some instances primary data have been col-

lected for these methodological inquiries and in other cases secondary materials are used.

The program of Drs. Yarrow, Burton, and Campbell has grown out of their work in the area of family and child development. It consists of a series of interlocking studies that have now been in progress about five years. Essentially, these studies aim to evaluate the correspondencies among sets of data gathered by different methods. Indirect techniques—i.e., those based on verbal reports—and direct observational techniques are being studied. A number of conditions which can influence the validity and reliability of interview and observational data have already been reported in previous annual reports. During the past year work has continued toward the identification of further relevant conditions. Thus, in interviews with mothers it was found that they could report with a richness of detail about the disciplinary techniques they use with their children. When questioned about the techniques they employ to praise or reward their children, however, the responses were quite barren and restricted in content. This is despite the fact that it is known through observations of mother-child interaction that techniques of reward are just as varied as disciplinary measures. A second study in this program focused on the potential biasing effects of experimenter's hypotheses upon the observations of behavior. As part of another inquiry (Project Number M-S-D-22), comparisons were drawn between the observations of a child's behavior made by the experimenter and by uninformed observers. A small but persistent tendency was found on the part of the informed observer to record behavior in line with the hypothesized experimental effects.

In another inquiry, interviews are presently being conducted with mothers concerning certain parameters of their children's behavior; these interviews are followed by direct observations of the same dimensions of behavior. Comparisons will be made of the data yielded by the two techniques. Over all, the various parts of this inquiry are building upon each other in a way that portends a body of work that is bound to have an incisive impact on research in family and child development for years to come.

Dr. Morris Rosenberg has been devoting part of his work to methodological problems, particularly as they occur in survey research. Typically, the survey researcher, following generally accepted conventions of data analyses, treats his data without a great deal of awareness of the operations he is performing. It might generally be said of Rosenberg's work in this area that he is attempting to codify the procedures employed in survey analyses and to specify the kinds of results yielded by different analytical procedures. No new data are being collected for this work; rather, data both from studies previously done by Rosenberg and others, are being re-examined. Some of this work will probably go into a revised edition of *The Language of Social Research*, a widely used methodological book co-edited by Rosenberg in 1955.

Finally, Dr. Harry Scarr is engaged in two inquiries having methodological significance. The first of these entails the difficult problem of how to deal with changes through time. In order to assess realistically and accurately the effects of social experiences on personality and behavior, it is necessary to devise ways to relate longitudinally specific experiences to the particular behavior or personality characteristics the experiences are thought to be modifying. Most existing techniques simply make comparisons at two points in time to determine if change has occurred and then look also at intervening experiences that occurred within the given time span. The drawback of such techniques is that they can only assert that events occur simultaneously; they cannot empirically demonstrate a cause and effect relationship between these events. Scarr, as principal investigator of changes among Harvard University students during their four years of college, is in an excellent position to deal with this critical problem. The data collection will be completed in the fall of 1965, at which time analyses will begin. However, he has been able to construct an analytical model for the study. Simply, this model provides for the determination of the structure of relationships of variables through time, thus permitting changes and their antecedents to be identified by alterations in this structure.

His second methodological study is also tied

to a substantive problem, in this case involving social values. To date, he has reviewed empirical studies of values that have been reported over the past twenty years. One of the striking findings of this review is the lack of consensus in the conceptualization of value systems and the heterogeneity of the instruments that have been employed. Scarr intends to take existing instruments used for the measure of values, identify their common dimensions and reduce them through factor analytic techniques. This would serve both to delineate the prominent theoretical systems of values and to create new and more manageable instruments. Following this, he plans a series of studies on the formation, maintenance, and change of values.

LABORATORY OF NEUROPHYSIOLOGY

The Section on Limbic Integration and Behavior is continuing its brilliant, comprehensive, and thorough work on brain physiology, anatomy and behavior. An ingenious experiment designed to locate and study units responding to noxious stimuli in the posterior thalamic complex and midline and intralaminar thalamic complex has been successfully applied in the squirrel monkey. This is a significant advance in both technic and results. This is being continued using the sensory division of the fifth nerve as activating system. The fornix tracts are also electrically stimulated to examine the role of limbic system in modulating sensory induced activity in the thalamus.

The general examination of photo-neuro-endocrine effects is being continued. An improved Nauta-Gygax stain has revealed lateral geniculate fibers in the posterior hippocampal gyrus. Sociosexual studies are being continued to determine the neuroanatomical substrate of the mirror display phenomenon. General social studies on aggression, social grouping, and eating habits are being continued.

The role of the membrane in our thinking about the physiological boundaries between cells, as well as between compartments within cells, is in a state of flux. In some cells it is easier to move material from inside one cell to inside its neighboring cell than it is to pass material out of the cell into the surrounding fluid. Electron micrographs show a sharing of the

plasma membrane by the two adjacent cells when such easy transfer between cells can be demonstrated. Thus the cell, by itself, has lost a bit of its independent identity. But our thinking about the cell is made even more complex because the boundary between what one should consider as inside and outside the cell is complicated by the presence of compartments within the cell that seem to be continuous with the external fluid. One such "intracellular" space is the transverse tubular system (T-system) of muscle fibers. These structures are believed to play an early role in the mechanism of the coupling between the electrical changes which are generated on the outside surface of a muscle fiber and the contractile apparatus within the cell. The **Section on Membrane Physiology** has been able to find methods for altering the size of the T-system, although they do not understand clearly the mechanism of the changes in size. Nevertheless, the electrical effects of the changes in size correspond well with their scheme to describe the function of the T-system. It is hoped that their scheme will provide a prototype for similar compartments that are found in other cellular systems, such as the cells of the brain.

Progress is being made on problems of blood-brain barrier and changes of neuronal polarization as a function of respiratory acidosis. New technics have been developed for making improved pH, CO₂ and O₂ electrodes. Measurement of end-tidal CO₂ has been improved. It has been ascertained that there is a distinct species difference between rabbit and cat regarding "steady" potential changes in the brain which accompany respiratory acidoses, which observation clears up some of the conflicts in the literature. It has been determined that the potential change shows only small gradients throughout the brain above the brain stem, but that it is so altered by hypoxia and deep anesthesia that doubt is raised that blood-brain barrier or blood-extra-cellular fluid barrier mechanisms are the source.

The technical staff is continuing with new developments in instrumentation. These include two transistorized amplifiers and stimulators, telemetry devices, and improved methods for calibrating and using thermocouples and thermistors.

LABORATORY OF PSYCHOLOGY

Only an inkling of the contexts for the individual projects is possible in the following descriptions. Each is part of a structure of theory and a progression of empirical findings. For many, the preliminary explorations to determine the most feasible approach have been completed and the experiments which can be done with presently available apparatus and subject populations are in progress. But if a behavioral principle is to be generally valid it must be tested in more than one kind of situation with more than one kind of subject sample, and its ramifications must be pursued through increasingly intricate combinations of variables. Consequently, the scope of a number of the investigations is being extended to wider subject populations, in some instances to populations outside of the American culture. And each Section is devoting a considerable portion of its efforts to the development of its technological capability for coping with complex problems of measurement and data acquisition.

Section on Neuropsychology

Four remote brain-stimulation systems were tested and evaluated on monkeys with implanted electrodes. Two of these systems (one developed for this Section by Johns Hopkins Applied Physics Laboratory, and the other by General Electric) are now completely operational. Both units have certain limitations, however, which are being overcome in two newer units. One of these (developed by Gulton Industries) permits long-range stimulation and remote switching between electrodes, capabilities which are important for field work. The other (General Electric's Mark II) has both of the foregoing features, and, in addition, permits remote control of all stimulus parameters. At this point, the latter system appears to be the most promising one, though final evaluation must await further development and testing.

The technique of measuring the impedance of neural tissue, which has proved to be a useful adjunct to stereotaxic methods for localizing brain structures, has also undergone further development. New data indicate that the reactive component of impedance provides

a better index of electrode location than does the resistance component, and that the optimum frequency of the measuring sine wave lies between 25 and 50 KC. Further refinement of this experimental tool could lead to its adoption as an aid in human neurosurgery.

In addition to these improvements pertaining to electrode implantation and stimulation, developments by others in the application of focused ultrasound have led to an improved method for producing subcortical lesions. Because this is a trackless method, it eliminates incidental damage due to electrode implantation as well as the dangers associated with it, such as current leakage, hemorrhage and edema. To determine whether focused ultrasound will produce the type of large subcortical lesion most useful for current neuropsychological investigations, an exploratory study was undertaken in collaboration with Dr. P. P. Lele of the Massachusetts General Hospital in Boston. The results were sufficiently encouraging to warrant the purchase by this Section of an ultrasound lesion generator.

The more substantive aspects of the Section's research this year fall into three major categories:

Cortical Mechanisms in Sensation and Perception

VISION. Previous experiments with monkeys have provided indirect evidence that the prestriate cortex is an essential relay in a pathway linking the striate area with the inferotemporal "visual" area. The major difficulty for this hypothesis has been the repeated failure to obtain severe and lasting visual deficits after bilateral prestriate lesions. New evidence has indicated a possible resolution of this difficulty. A study of the effects of partial inferotemporal lesions revealed that removal of only a small cortical strip at the posterior extremity of the temporal lobe produced a moderate degree of deficit in visual discrimination. This posterior temporal strip, in contrast to the more rostral portions of the inferotemporal "visual" area, is now known to receive direct projections from the striate cortex. The apparent ineffectiveness of prestriate lesions may be attributable to the failure to include in the

removal this small but potentially important temporal sector of the striate-prestriate projections. Studies are now under way to test this possibility.

The recognition that there may be not one but two foci for visual functions in the temporal lobe, a posterior and an anterior one, could help also to clarify the nature of the visual deficit produced by large inferotemporal lesions. Thus, selective removal of each of these foci could perhaps lead to a separation of visual deficits which have previously been confounded.

Analysis of the visual deficit produced by temporal lobe lesions is also being carried out on neurosurgical patients. Thirty cases with unilateral temporal lobe excisions (performed by NINDB surgeons for the relief of epilepsy) have been recalled to the Clinical Center for extensive visual testing. Preliminary comparison with normal control subjects suggests that the patients' asymmetrical impairment of recognition in the two fields (i.e., greater impairment in the field contralateral than in the field ipsilateral to the removal) is not associated with an asymmetrical defect on basis sensory tests. This tentative finding supports a conclusion derived from work on monkeys that the visual disturbance after temporal lobe damage is not reducible to visual field or acuity defects.

AUDITION. Research on neural mechanisms in audition has been impeded for many years by the lack of an efficient method for training monkeys to perform even simple auditory discriminations for food reward. This problem has been solved in the past year by utilizing an instrumental conditioning technique which was first developed for use with dogs. With this technique it has been possible not only to train monkeys in frequency and in directional discriminations, but also to obtain discrimination thresholds quickly and reliably. A preliminary result using this method to study the effects of cortical lesions on auditory functions indicates that a marked increase in auditory thresholds may be produced by a lesion of the superior temporal convolution which spares the main auditory projection area.

SOMESTHESIS. The initial aim of these studies was to find the locus of lesion which would

disrupt the memory trace for previously learned tactal discrimination habits. The method was to train one hand and test for retention (i.e., transfer) with the other. In the first phase, it was shown that normal animals transferred almost perfectly, whereas animals with section of the forebrain commissures prior to any training showed no transfer. However, commissurotomy subsequent to training of the first hand also caused a decrement in transfer. These results indicated that recall was in part dependent on a transcallosal contribution by the "trained" hemisphere and thus made it possible to ask whether or not there was a focal area within this hemisphere essential for memory. The second phase of the experiment investigated the effects of two large and complementary lesions of the trained hemisphere, sensorimotor (SM) and nonsensorimotor (NSM). It was expected that recall of the tactal habit would be disrupted by the SM lesion but not by the NSM lesion. Contrary to these expectations, both lesions appeared to produce the full decrement.

To investigate the hypothesis that the SM removal resulted in a true memory loss whereas the NSM removal interfered with transfer performance in some other way, additional groups of animals were tested for original learning with the hand used for the transfer tests in the previous experiment (i.e., the hand ipsilateral to the removal). The results showed that, in comparison to normal performance, the SM lesion produced marked impairment of the ipsilateral hand in learning tactal discriminations and in roughness thresholds, but that the NSM lesions had no effects.

These experiments have thus revealed two important phenomena, each of which is under further study. First, it is clear that the transfer decrement following the NSM lesion cannot be ascribed simply to impaired performance with the ipsilateral hand; whether this lesion produces a true memory loss, however, is still open to question. Data are being gathered to assess the effects of the nonsensorimotor lesion, and of subdivisions of this lesion, on tactal performance with the contralateral hand. Second, the transfer decrement after the SM lesion may be explained by the associated dis-

crimation loss; however, this discrimination loss is, itself, of considerable interest, since no deficits after lesions confined to the ipsilateral hemisphere have been demonstrated before. To pursue this finding, which bears directly on the problem of the cortical representation of somesthesia, a study is in progress on the effects of partial lesions of the sensorimotor regions on somatosensory thresholds of the ipsilateral hand.

The effect of cerebral lesions on tactal functions is also being studied in man. Sixteen patients admitted to the Clinical Center for surgical therapy of Parkinson's disease (lesions of n. ventralis lateralis) have been tested pre- and post-operatively. This nucleus is the origin of the major afferent system to the motor cortex, a region which may serve somatosensory functions as well. Quantitative tests of punctate tactal sensitivity, two-point discrimination, and point localization on the face, hands, trunk, and feet are applied in order to determine the nature and distribution of the impairment. Preliminary results indicate elevation of thresholds in some patients but not in all. Bed space in NIMH has been secured to extend the patient's stay by five days. It is hoped that this additional time will increase the number of subjects who can participate.

Cortical Mechanisms in Problem Solving

Whereas the work on sensation and perception pertains mainly to the posterior and central regions of the cortex, the research on problem solving pertains principally to the frontal regions. New insights into the nature of frontal lobe function in monkeys have emerged from the study of the effects of partial frontal lesions. Indeed, it seems now that many of the perplexing problems in this area of investigation have stemmed from past failure to recognize fully the functional heterogeneity of frontal cortex.

Earlier experiments had led to the proposal that the deficit produced by frontal lesions on spatial delayed alternation was related neither to the delay nor to the reversal feature of the test, but rather to its spatial feature. According to this hypothesis, eliminating the spatial feature, by testing for delayed alternation be-

tween objects rather than between positions, should result in the elimination of the impairment. The initial attempt to confirm this prediction was unsuccessful in that frontal lesions were found to produce equally severe deficits on both tests. Following this first attempt, however, data were obtained from other experiments which suggested that the seemingly similar impairments on the two tests of delayed alternation may have resulted from two quite different defects, each produced by a different segment of the total lesion. This modification of the original hypothesis recently received strong support from a second attempt to dissociate the effects of frontal damage on the two forms of delayed alternation. The spatial version of the test was affected more severely by dorsal than by ventral frontal lesions, whereas on the nonspatial version the relative effects of the two lesions were reversed. The confirmation was not complete, however, since the dorsal frontal removal did produce some impairment on delayed alternation between objects. Whether or not further delimitation of the dorsal lesion will completely eliminate this nonspatial impairment remains to be determined.

A second prediction stemming from the revised hypothesis of frontal lobe function has also received confirmation. If the deficit following the dorsal lesion is related to a spatial factor, it should be possible to detect the deficit utilizing a spatial test from which the delay and reversal features have been removed. Comparison of the effects of selective frontal lesions on such a task has in fact shown that the dorsal removal causes an impairment, whereas the ventral removal does not. This is particularly noteworthy in that it is the first demonstration that the specific lesion responsible for the classical delayed-response deficit will impair performance on a task which does not involve "recent memory."

Although the impairment following dorsal frontal damage has been tentatively related to the spatial aspect of certain problem-solving tasks, the nature of the defect is unclear. Whether it is a form of spatial disorientation or a difficulty in response discrimination now becomes a question for future studies.

Some progress has been made on the parallel question of the nature of the defect produced

by ventral frontal lesions. An earlier series of experiments had suggested that animals with this lesion are deficient on any task requiring suppression of a dominant response tendency. In addition to reversal tasks, this category includes tests involving object preferences and aversions, response to novelty, and differentiation, which requires the animal to respond actively to a positive stimulus and to withhold response to a negative one. Following an initial experiment demonstrating that impairment in auditory differentiation was in fact produced by ventral but not by dorsal frontal lesions, a second experiment indicated that a removal confined to the lateral sector of the ventral cortex produced an initial post-operative impairment comparable in severity to that following the total ventral lesion; a removal limited to the medial sector, on the other hand, had no effect. Training was continued until the animals with both types of partial ventral lesions attained criterion performance, at which time their thresholds for frequency discrimination were determined. In contrast to the clear separation between groups observed on the initial problem, no group difference could be detected on the threshold measure. It appears unlikely, therefore, that the initial impairment in differentiation performance following the ventrolateral lesion is due to a loss of discrimination ability.

Cortical-Subcortical Relations and the Regulation of Behavior

Although much has been learned about the cortical factors significant in discrimination and problem solving, little is known about the way in which these factors actually regulate behavior. The classical view that behavioral responses are produced by cortico-cortical influences playing upon the precentral motor area has received little support in recent years. The possibility that the behavioral measures which have been employed to test this concept have not been sufficiently sensitive has led to new experiments to investigate the issue further.

A situation designed to measure reaction time has yielded reliable baseline reaction

times of 250 msec. and below. In another situation, designed to measure errors in reaching induced by ophthalmic prisms, animals are adapted with one eye-hand combination and then tested for transfer of the prism-adaptation to the other eye. In both situations, the primary aim is to restrict the visual input to one hemisphere, require a motor response from the other, and then test the effects of section of the cortical commissures. Preliminary results indicate that neither reaction time nor reaching accuracy is altered by the transection. While further work with both techniques is planned, the negative evidence thus far obtained from these experiments, together with some positive findings obtained in experiments involving subcortical lesions, focuses attention on cortical-subcortical relations.

The studies on subcortical structures have been concerned mainly with the striatum and the limbic system. The recent discovery of an extensive, topographically organized system of direct cortico-striatal connections has suggested that the striatum may be the first link in a major pathway providing for the cortical regulation of lower centers. This hypothesis is supported by some of the results from this project. Thus, a lesion confined to the anterodorsal portion of the head of the caudate nucleus, the region to which the dorsal frontal cortex projects, has been shown to produce the same effect as a dorsal frontal lesion—impairment on spatial delayed alternation. Similarly, a lesion confined to the tail of the caudate, which receives projections from the inferotemporal cortex, was found to produce, just as inferotemporal lesions, impairment in visual discrimination. An attempt to reproduce by striatal damage a third type of cortical effect, the perseverative impairment following central frontal lesions, has not yet been successful. This negative result has led to a neuroanatomical study designed to provide more detailed information concerning the efferent projections of ventral frontal cortex.

Additional evidence that the cortex and striatum are functionally related has been derived from the studies on prism adaptation. A survey of the effects of various cortical lesions has shown that the process of adaptation is im-

paired only by extensive prefrontal removals. Significantly, a similar impairment is produced by lesions of the head of the caudate nucleus.

The other major forebrain system which receives direct input from the cortex, and which must therefore be considered another potential relay for the cortical regulation of behavior, is the limbic system. Studies are currently under way to determine the behavioral effects of amygdala, hippocampal, and septal lesions, particularly on the functions served by ventral frontal cortex. These experiments are still in an early stage, though preliminary results with septal lesions have revealed deficits in both passive avoidance and extinction of an instrumental response, two of the deficits which have previously been observed after ventral frontal lesions in monkeys.

Section on Aging

The Section on Aging was formerly divided into two units, one on Psychophysiology and one on Higher Cognitive Processes. During the present year, however, Dr. James Birren, the former Chief of the Section, transferred to the National Institute of Child Health and Human Development, and the Unit on Psychophysiology was terminated. The Unit on Higher Cognitive Processes has continued its studies of heuristic processes in the aged, schizophrenics and normals.

Psychophysiology

Dr. Jakubczak has reported on a study of thermoregulation and heat rewarded lever pressing behavior in contrasted age groups of rats maintained in an ambient temperature set at 2° C in a refrigerator. No age differences were found with respect to the rate of decrease in rectal temperature, the length of delay in initiating a steady rate of responding to the lever that produced a warming stimulus, or the steady rate of responding. A review of the influence of hormones on the age related mating behavior was prepared by Dr. Jakubczak in connection with a study in which he found that castration did not influence the running behavior of Syrian hamsters.

Higher Cognitive Processes

Eleven families having identical twins, one of whom had been classified as schizophrenic, the other being undiagnosed, were tested on a set of problem-solving tasks monitored by a machine called HEPP (Heuristic Evaluation and Problem Programmer) that was developed in this Laboratory. The analyses that have been completed indicate, in addition to marked heuristic impairment in some of the schizophrenics, a wide range of individual differences in heuristic ability within all sub-groups of the families on even the simplest of problems. The principal heuristic deficits identified may be characterized in the following manner: 1) Sequential inquiries were poorly organized so that the cognitive strain associated with a solution effort was much greater than it needed to be; 2) Poorly adapted behaviors were allowed to persevere without exploring obvious alternatives; 3) The questions reflected by the subjects' operations were so specific that the information elicited was near minimal; 4) Quite direct implications of information elicited were not used; 5) Failure to use negative information prolonged the use of inefficient procedures.

A group of normal control subjects (college students) has been tested on a set of mixed three element conjunctive HEPP problems. Using a procedure whereby displays are automatically read into the apparatus, efforts were made to teach strategies for solving these complex problems. Although the subjects were told that a number of solution strategies would be shown, only a few changed their previously developed solution method, inefficient, confusing, and inelegant as it was. There was evidence of a surprising inability to invent techniques for reducing cognitive strain or to learn from the displays rather obvious methods for doing so.

In order to secure an opportunity to observe the interpersonal relations between members of test families while engaged in a cooperative solution effort, HEPP was redesigned to operate alternately from two subjects' panels each located in a separate test cubicle. The subjects can communicate over a public address system and are free to question, advise or criticize one another while working. It is anticipated that this study will provide evidence of dominance,

submission, aggression and cooperation between pairs of individuals as well as information concerning the effects of these factors on heuristic performances. A preliminary procedure has been tested on one pair of twins and was found very encouraging.

This unit has also set up and administered an automatic data processing facility operating an IBM 1620 computer and accessory equipment. The facility services about 10 investigator-programmers from NIMH who are assisted by one professional programmer, Mr. John James. The system is in operation about 350 hours a month. During this first year of service, the use of the facility has increased more than was anticipated and it is expected that the work load assigned to it will continue to increase for some time. A disk drive attachment with a Monitor I system has, therefore, been requested to expedite compiling and execution of programs. It will probably be installed in June. Mr. James is attempting to develop a generalized programming language that can be automatically translated into any of the languages available to the investigator-programmers, e.g., Fortran, SPS, LAP (LINC assembly language). This will enable the user to program for several assemblers and machines in a single language. If successful, it should greatly decrease the time and effort the investigator must devote to programming. In general, computer technology is developing so rapidly within the Laboratory that it is now expedient to consider acquisition of a system that is more powerful and versatile than the IBM 1620 configuration that will be in operation in June.

Section on Perception and Learning

Environmental and Genetic Variables affecting Biological Systems

An extensive computer analysis of data collected on the domesticated Norway rat between 1954 and 1962 is providing an efficient means of specifying the occurrences of basic behaviors. These data involve nearly 200,000 discrete behavioral states for which starting and terminating times were recorded. For experimental and conceptual purposes, the behavior of the rat has been divided into six categories: locomotion, eating, drinking, grooming, and

short and long periods of sleep. The sequences and durations of these behaviors can be described by three probabilities, provided the environment remains constant.

At any moment in time each behavioral state has a specific probability of starting, given that it or another behavioral state has just terminated. Once started, any behavioral state has a particular probability of stopping within the next minute regardless of how long it has already lasted, given that a hypothetical circuit is open permitting the arrival of a neural signal which terminates the behavior. The third probability is the probability that this circuit is open. These probabilities vary on a 24-hour cycle for each behavioral state but have distinct patterns for different behavioral states.

The current analyses suggest that it may be possible to describe an animal's total behavior in terms of these probabilities, with a motivational aspect associated with the probabilities of starting and continuing a behavioral state and an emotional aspect associated with changes in the probability of starting or continuing.

Perception

This year has been devoted primarily to further investigation of a problem of time and motion perception in schizophrenic patients, normal perception of object-size, and the development of a new apparatus system for the control and recording of variables in perceptual experiments with human subjects.

Over the past several years a judgmental response-time task has been developed which provides a quantitative measure of the ability of schizophrenic patients to discriminate the spatial and temporal parameters of a visual stimulus-display. Previous studies with unselected groups of schizophrenics have shown that about one-third of the patients develop lengthened absolute response-times beyond the range of those for normal subjects while at the same time maintaining a very high correlation with the correct response-values. This phenomenon appears with repeated testing over a number of sessions and is related only slightly if at all, to simple reaction-time or to the subject's initial response-level. An analysis completed this

year revealed a significant association between this increase in response-time and a progressive change in the judged duration of a 2-sec. interval. Perception of stimulus motion and distance appears to be normal, but the subjective duration of a short time-interval apparently undergoes a progressive contraction for these patients when they are tested repeatedly in the same experimental task.

Much current theory concerned with the fundamental nature of visual perception is based on the assumption that elementary sensory information is elaborated into a complex perceptual result through the operation of an integrative cognitive process. In the perception of the size of an object, for example, it is assumed that apparent size must basically vary inversely with distance, since the size of the image on the retina does, and appreciation of true object-size must be arrived at inferentially by taking cues to distance into account. An evaluation of this viewpoint has been pursued in a number of experiments in this laboratory. In the most recent the prediction was derived that size judgments would vary as a function of distance only when the judgments made at different distances are not independent of each other. This was the result obtained, and it indicates that object-size is basically perceived accurately and variation with distance arises as an error factor due to the intrusion of an attitudinal bias. This conclusion is approximately the opposite of the traditional viewpoint of perception as a cognitive process, at least as far as the perception of size is concerned. Which of these alternative conceptualizations is correct makes a great deal of difference to an understanding of perceptual development, both normal and abnormal.

A punched paper tape system for more automatic programming of experimental stimulus-displays and recording of subjects' responses has been designed and partially implemented. The intent of the design is to achieve an efficient means of presenting complex series of experimental conditions and recording of data in a form suitable for immediate computer analyses as an experiment progresses. The system is a general one, capable of programming any experimental apparatus which can be controlled electrically or electronically.

Section on Early Development

Children's Perceptions of Parent Behavior

The goals of Dr. Schaefer's studies are to develop more adequate concepts and methods for investigating the interrelationships between parent and child behaviors and to integrate the concepts into conceptual models. Progress has been made in this continuing program toward a comprehensive understanding of parent-child relationships. Eventually the methods should be useful in diagnostic studies and in mental health screening.

In collaboration with Dr. Nancy Bayley, Dr. Schaefer is collecting retrospective reports of parent behavior from 37-year-old subjects who had been studied earlier in the Berkeley Growth Study. Their retrospective reports are being related to observations of their mothers' behavior with them when they were zero to three years of age and with interviews with their mothers when they were nine to fourteen years of age. Analyses of these data will provide information on the validity of retrospective reports of parent behavior.

Another facet of the research on children's perceptions of parent behavior is an investigation of the cross-cultural generality of the organization of these perceptions. In collaboration with a French-speaking Belgian student, the Child's Report of Parent Behavior Inventory was translated and administered to approximately 200 Belgian children. The scales yielded reliable measurements and three major factors of parent behavior—Acceptance vs. Rejection, Psychological Autonomy vs. Psychological Control and Lax Control vs. Firm Control—that replicated the spherical conceptual model for parent behavior that was derived from American data. Investigators in several countries—India, Hungary, Czechoslovakia, The Netherlands, and South Africa—have expressed interest in collaboratively determining the cross-cultural generality of this organization of children's perceptions of parent behavior. Such studies may well lead to cross-culturally valid generalizations about the interrelationships of parent behaviors and child behaviors.

Dr. Schaefer is continuing his analyses of parent and child behaviors into discrete com-

ponents and his integration of these components into conceptual models for parent behavior and for child behavior. Hierarchical orderings of parent and child variables at different levels of concreteness vs. abstractness and specificity vs. generality are leading toward taxonomies of parental behavior and child behavior variables. The development of ordered sets of interrelated concepts has permitted the construction of spatial maps of parent variables and child variables, and more comprehensive sets of scales for measuring these variables are being developed.

Progress has also been made in studying the relationships between the parent behavior and child behavior models. In a study of 154 male adults, retrospective reports of maternal and paternal behavior and self-reports of adjustment were collected. Three major dimensions of parent behavior were correlated with three major dimensions of self-reports of adjustment. Two parent behavior factors—Rejection and Psychological Control—were significantly correlated with self-report of adjustment factors of Severe Maladjustment and Introversion. Significant parent-child correlations were not found for the adjustment factor of Neurotic Repression nor for the parent factor of Lax Control vs. Firm Control. Paternal behavior was more highly related to maladjustment for the factor of Rejection while maternal behavior was more highly related for the factor of Psychological Control.

Intellectual Stimulation of Culturally-Deprived Children

Dr. Schaefer has also undertaken the preliminary planning and organization for a study to evaluate the hypothesis that environmental stimulation during the period of early verbal development can raise the level of intellectual performance of lower socio-economic status children. Recent research suggests that various forms of verbal stimulation during this age period can improve intellectual functioning, and a determination of the effectiveness with which this might be done has important implications for basic research on intellectual development as well as for social action programs.

Learning in the Neonatal Dog

Using ingestive behavior as the index of behavioral change, Dr. Stanley has reported major findings on the development of social behavior in juvenile dogs, completed the design of a fully instrumented apparatus for maintaining newborn mammals and studying their behavior. He has also prepared a draft summarizing his extensive research on early motivation and learning.

QUANTITATIVE PROPERTIES OF EARLY LEARNING. Until recently it was believed that puppies less than two weeks of age either could not learn, or, if they did learn, retained what they had learned only briefly. Previous research, done here and elsewhere by Dr. Stanley, had established that the neonatal puppy could learn and retain what he had learned from day to day. During the past year, further research on this problem has clearly established for the first time that true stable conditioning does occur in neonatal puppies. Under precise conditions, learning in the neonatal puppy is rapid rather than slow, especially when conflict is not involved. The findings further suggest that research on the human infant must be greatly improved in precision to permit adequate testing of the learning abilities of human newborns.

SOCIAL BEHAVIOR. Juvenile and adult dogs are unique in the ease with which they develop a strong attraction to man. This feature of canine behavior provides an excellent experimental tool for analyzing the reinforcers (rewards) inherent in social interactions where apparent emotional dependence is involved. Two experiments done elsewhere by Dr. Stanley were reported in the literature this year. The first demonstrated that mere presence of a seated, passive person acts as a reinforcer to maintain juvenile puppies' approach behavior; the second experiment demonstrated that prior association of people with feeding is not necessary for contact with a passive person to have a rewarding effect on puppies' behavior. This work has also suggested a new explanation of fear of the strange, or unfamiliar; namely that such fear is a function of the absence of customary, positive, sensory reinforcers.

Research on canine social behavior will be resumed when the necessary research facilities become available to NIMH at the Poolesville Animal Center.

AUTOMATION OF APPARATUS. One of the main limitations of precise research on the behavior of newborn mammals, including human infants, is the absence of apparatus specifically adapted to the measurement and control of behavioral systems which are efficient early in life, as, for example, suckling. This limitation has required much handling of subjects and arduous work schedules for professional and technical personnel. Significant advances in automating research procedures have been made during the past year. It is hoped that full instrumentation of research procedures will be possible during the coming year.

The Impact of Learning and Environment in Child Development

The major emphasis of Dr. Gewirtz's work has been on the human learning process in social contexts, especially on the mechanisms of adaptive and social learning in the early phases of life. During the past year this program has focused on determining the relationships between selected infant behaviors (smiles, vocal and similar responses), which would reflect social responsiveness and the quality of adaptation to the maintaining environment, and theoretically important differences in environment and experience.

For the first project, some 600 infants were observed in the first 18 months of life in residential institutions, in day-care centers, in single- and multiple-child families, and in the unique kibbutz environment. The results for differences in the forms of the age curves for smiling among the several environmental groups are different in important ways from results already in the literature. The reasons for these discrepancies are being explored. They imply that certain contextual conditions have not been controlled in previous studies. Dr. Gewirtz is currently processing the smile curves of individual infants in the several environments. The greatest number of these appear to follow an exponential form in their decline patterns.

Stimuli provided by the environment to the infant, the infant's behaviors, and the relationships between the two are in the process of being catalogued for 108 infants. This research program has involved an extensive amount of preparatory work, but considerable progress has been made during the past year on the processing of the data collected and this phase should be completed in another year. The results will provide considerable information on the impacts of widely differing types of environmental conditions on children's behavior systems in the formative period of their lives.

Work on a third series of experiments has been completed. These experiments involve a pre-test condition providing school children with different numbers of a social stimulus class (non-contingent on behavior) and then checking the saliency (or reinforcing effectiveness) of that stimulus class in learning tasks. It has been found that the more frequently stimuli are earlier presented ("satiation"), the less effective they are in the test. Further, temporal conditions of recovery from this satiation are being investigated: more recovery has been found after longer delay intervals between treatment and test and less recovery after shorter delay intervals. In this way a number of contextual conditions for the saliency of social stimuli controlling behavior and learning have been investigated. Up to now, this type of lawfulness had been identified mainly with appetitive stimuli and typically with lower organisms.

Section on Personality

The variety of studies which are being carried out in this Section have a common focus at a broad conceptual level. The three current research programs are all concerned with cognition and its interrelationships with other psychological processes. The major project encompasses the broadest view of cognition. In this program the social and personality determinants of scientific creativity are at issue. The second research program focuses experimentally on the role of cognitive mediation at a strategically chosen pre-conceptual period in children. The third program is concerned with some ba-

sic psycholinguistic issues in the relationship between cognition and speech.

Creative Functioning

This research program has grown out of two interrelated purposes: 1) a basic interest in issues of personality theory, and 2) an interest in specifying and examining the creative process and some of its psychosocial determinants. In the Creativity Project these two interests become tangent in theories which postulate childhood determinants of adult functioning. Current longitudinal studies of non-deviant people strongly suggest that our concepts of personality development stand in need of considerable revision. Psychopathology is not a necessary consequence of severe strain and stress during childhood. To the contrary, there are some indications that certain early stresses and painful, confusing experiences have a constructive effect on those who survive them.

The present study of effectively functioning and potentially creative young scientists may provide additional data relevant to this central problem in personality theory. The overall research design includes three kinds of inquiry: 1) the retrospective study of influential factors in the personality development of a group of potentially creative young men; 2) a predictive study regarding their creative performance; and 3) an investigation of personality change between adolescence and maturity.

PERSONALITY FEATURES OF POTENTIALLY CREATIVE ADOLESCENTS. This comprehensive study is the foundation for the research program in creativity. The original sample was a group of 536 subjects chosen from participants in the 1963 Science Talent Search. All have demonstrated a high level of scientific competence but were further subclassified into three groups: 112 rated High Creative, 137 Middle Creative, and 287 Low Creative.

Measures of intelligence, personality and social history variables were obtained and have been analyzed with the following results: 1) Some personality characteristics are related to early scientific attainment. The relation is generally linear for measures of cognitive style and generally curvilinear for measures of psychological complexity and personal tension. 2)

Group averages appear to mask subgroup patterns, in that further subdivision reveals a variety of personality patterns associated with similar levels of achievement. 3) Comparisons with studies of adult creative groups suggests that this younger population is similar to them with regard to cognitive style variables but dissimilar with regard to personal and social variables.

A second assessment of these students was made during 1964 when most of them were sophomores in college. Analysis of these data is in progress. Preliminary results suggest that the three subgroups do not differ in college scholastic attainment, preference for science as a career choice, or general satisfaction with college.

A replication of this study has been launched, using the 1965 Science Talent Search applicants, selected according to the 1963 criteria. Continued periodic follow-up assessments are planned for both samples, and certain subgroups will be selected for personal interviews and experimental studies.

SOCIALIZATION FACTORS AND CREATIVITY. This aspect of the Creativity program is the investigation of birth order and religious background as they may relate to creative potential. The data have been drawn from the social history questionnaires completed by the 1963 sample of Talent Search students. Relevant statistical analyses are compared and contrasted with published data from previous studies in order to synthesize the available evidence.

The data do not support the widely-held hypothesis that birth order and creativity are related. Previously reported data demonstrating a predominance of first-borns among creative men may be accounted for by an artifactual tendency for first-borns to be over-represented generally among students of high scholastic attainment.

The relationship between religious background and creativity, however, does seem to be supported by these data. Since this is merely an actuarial finding, further work is under way in order to delineate patterns of child-rearing practices which may be associated with religious affiliation.

PARENTAL INFLUENCES ON PERSONALITY DEVELOPMENT OF THE POTENTIALLY CREATIVE SCIENTIST. This is a retrospective investigation of parental influences which may differentiate more and less creative young scientists. For this study the 1963 and 1965 samples are combined, yielding a total of 980 subjects: 190 high, 250 medium and 540 low on rated creativity.

All subjects will complete a Parent-Child Relationship instrument which has been developed. Five dimensions have been defined: 1) Parental values, 2) Sources of parental influence, 3) Consistency of parental values, 4) Modes of training, and 5) Modes of reward and punishment. These dimensions will be measured by a total of 61 scales, most of which have been drawn from existing measures. Since this study is in the data-gathering phase no findings are yet available.

THEORETICAL STUDIES. Two additional studies are being carried out with the purpose of refining certain theoretical issues in the area of creativity. The first of these takes as its point of departure the question, Is there a creative personality? This study seeks to develop empirically meaningful criteria to evaluate evidence for the existence of a general personality pattern associated with creativity. The problem will be approached through a theoretical analysis of the implicit assumptions involved, a survey of appropriate statistical models, and, if it proves necessary, the development of new modes of analyses.

The second of these studies focuses on individual standards of evaluation as these may modify creative behavior. The experimental treatment involves reinforced discrimination training on sets of pairs of figure completion drawings. Each pair will contrast degree of some dimension: shading, for example, or action, or humor. The experimental measure will be the comparison of pre- and post-training figure completions done by the subjects to determine if their performance is modified in terms of those dimensions which were reinforced. The general hypothesis under test is that more and less creative individuals may differ more in response selection than in their repertoires of available responses.

Role of Various Response Systems on Conceptual Mediation

This research is concerned with the response systems essential for problem solving and creative insight, namely, those that allow the individual to treat physically dissimilar stimuli and dissimilar relationships as equivalent. The current work focuses on the role of nonverbal "coding" responses in this regard and on their interrelationship with verbal responses.

This program has selected as a strategic research locus the pre-conceptual child and, by "building-into" the child various response repertoires, attempts to determine which ones are necessary for mediating conceptual transfer and equivalence. During the past year the research has concentrated on the "middle size" concept, selecting for study those pre-school children who do not yet have the concept. Screening of some 350 nursery school children in Montgomery County revealed that 85% of those in the age range 3.4-3.9, 63% in the 3.10-4.3 range, and 46% in the 4.4-4.9 range do not yet have this concept. Interestingly, the figures for girls were lower than those for boys at each age level.

The experimental work completed thus far demonstrates that it is possible to train "no-concept" children to transfer in terms of middle-sizedness without benefit of any verbal mediating response. No-concept girls were better than no-concept boys in this regard and no-concept children in the oldest age-range were better than no-concept children in the youngest age range. Now under way is an attempt to determine the precise aspects of the training that were responsible for transfer. Intensive work will shortly begin with those children who failed to transfer, in order to uncover their specific deficiencies.

Effectiveness of Psychedelic Therapy (using LSD) in the Treatment of Hospitalized Alcoholics

In a preliminary study, volunteer patients in the Alcoholic Rehabilitation Unit at Spring Grove State Hospital were randomly assigned to experimental and control treatment conditions. The experimental treatment was three weeks of intensive psychotherapy surrounding

one all-day LSD session. The control group received the standard hospital treatment program.

All indications were that the experimental treatment program is safe. Statistically significant changes in a therapeutic direction were measured in the experimental group. These changes may generally be described as a decrease in depression and anxiety and the enhancement of independent functioning.

This project was terminated at NIMH with the resignation of the principal investigator December 31, 1964. It is being continued at Spring Grove under an NIMH sponsored grant.

Investigation of Some Formal Characteristics of Speech

The summary of this project has been incorporated with the summary on Human Communications in the Section of the Chief.

Research Directions

In general, the future course of the work of this section will be the extension and amplification of work summarized above. The Creativity Project hopes additionally to extend its investigations to include more intensive study of abilities and environment as these affect the development of creativity.

The course of Cognition Project is envisaged as a large scale expansion of its experimental program with pre-school children, most probably by means of a mobile testing unit in the form of a self-contained trailer.

Office of the Chief

Research in the Office of the Chief has been concerned not only with a number of basic problems in schizophrenia, psychotherapy, and communication, but also with analyses of the background and current implications of several central issues in the development of psychology as a science and a profession. In the latter category, an evaluation of clinical psychology in relation to the goals recommended for graduate training by the American Psychological Association in 1947 and an examination of the moral aspects of psychoanalysis were completed

this year. Developments in the more specifically delimited research projects follow.

Schizophrenia

PSYCHOLOGY OF SCHIZOPHRENIA. As part of a program of analyses bringing together an extensive series of previously collected experimental data, the Chief has derived a composite index for the Kent-Rosanoff Association Test. The index is based on a differential weighting of three kinds of response on the Association Test. Responses obtained from 200 normal subjects and 100 schizophrenic subjects were scored according to this method, and the means of the resulting distributions were found to be different at a highly significant level. Another part of this series was concerned with a comparison of the reactions of schizophrenic subjects with those of normal subjects to tautophone stimuli. The tautophone is a device for presenting a set of sound patterns repetitively. Under instructions implying that the sounds consist of meaningful but unclear speech, it provides a means of assessing apperceptive aspects of behavior. Analysis of formal characteristics of the responses indicated that the tautophone procedure has considerable power to discriminate psychotic qualities in the subject's manner of responding.

HEREDITY AND ENVIRONMENT. Dr. Rosenthal began the year by participating as a faculty member in the Institute on Behavioral Genetics at the University of California at Berkeley. He not only had occasion to evaluate and present a fairly inclusive summary of our knowledge about the etiology of schizophrenia, but to obtain firsthand reactions to this presentation of some of our most knowledgeable, genetically-oriented psychologists and psychologically-oriented geneticists. The range of thinking on the problem of heredity in schizophrenia has expanded to encompass the broad question of the relationship between human heredity and psychology in the light of evolutionary theory. It is too early to say if, or how far, this line of thinking can be developed, but schizophrenia must be considered in terms of this larger problem if it is to be adequately understood.

For most of the rest of the year Dr. Rosenthal has devoted his efforts to organizing two studies concerned with the relative contributions of heredity and environmental factors in schizophrenia. The first project takes advantage of a way of life unique to Israel, viz., the kibbutz. The question asked by the study is: Will the kibbutz familial and social organization be more or less beneficial to the child who has a schizophrenic parent? On the assumption that a group of schizophrenic parents has a higher genetic loading for schizophrenia than a group of selected control parents, the design means that two genotypes are being varied as well as two environments. This allows estimates of the proportion of the variance contributed by the assumed genetic variation and the proportion contributed by the heredity-environment interaction with respect to a number of personality characteristics and test behaviors of the children.

The second study begins with schizophrenic parents whose children were given up for adoption at an early age. Matched to this group are parents who have not been known to have any psychiatric disorder, but whose children were also given up for adoption at an early age. The two sets of children are then compared psychiatrically and psychologically, and control for psychiatric disorders among the adopting parents will be evaluated as well.

PSYCHOPHYSIOLOGICAL RESPONSIVITY. During the past year emphasis has been given in Dr. Zahn's studies to investigations involving the concurrent recording of the responses of autonomic variables such as skin resistance, heart rate, finger pulse volume, skin temperature and respiration to stimuli which may be considered as varying in meaning or in demandingness. Previously it was found that while acute and chronic schizophrenic subjects are respectively equal to or greater than normals in physiological responsivity to "meaningless" auditory stimuli they are significantly lower in physiological responsivity to the stimuli in a simple reaction time experiment. Additional analyses of the data have shown that the physiological responsivity deficits for the schizophrenic subjects are greatest under the conditions where their reaction time deficits are the greatest. Further, in both schizophrenic

and normal groups subjects with faster reaction times have the greater physiological responsivity. Measures reflecting the general level of arousal, however, show that for normals reaction time is correlated with a high arousal level while for schizophrenics high arousal is associated with slower reaction times. These results suggest an inverted-U model for the relation of behavior and arousal in schizophrenia and also suggest the necessity for distinguishing between general arousal levels and specific alerting systems.

Similar investigations are in progress with identical twins and their parents who have schizophrenic adopted children. Preliminary analyses of the data of seven twin pairs who are discordant for schizophrenia show that the relationships of physiological responsivity to the demandingness of the stimuli for the non-schizophrenic and schizophrenic twins are much like those previously obtained for unrelated normal and schizophrenic subjects, respectively. Reaction time differences show a similar relationship.

Another study, done in collaboration with Dr. Schoeler of the Laboratory of Socio-environmental Studies, investigated the influence of having a patient cooperate in a block design task with another "patient" (a confederate) as opposed to doing the task with just the experimenter present. It was expected that the effect of the other patient would be to lower performance and raise the level of arousal and that this would be more pronounced in the "sicker" patients. The results on performance show that, contrary to expectation, the performance level was improved by the introduction of the other "patient" and that this effect was more pronounced in the "sicker" group. The physiological data are currently being analyzed.

Much effort has been expended during the year in developing a system for automatically analyzing GSR data from these experiments. The system is now in operation using the LINC and IBM 1620 computers. This system detects, computes and records for further processing the amplitudes of specific and non-specific responses and the baseline skin resistance. The analysis of other parameters, such as latency and duration, is also possible.

ORAL FUNCTIONS. Dr. Bergman and Dr. Zahn have continued work on the study of oral functions. The original hypothesis underlying this study proposed that schizophrenics would significantly differ from controls on some oral functions. The first groups examined strongly supported the hypothesis, but subsequent replications have confirmed it only slightly. Interest has therefore shifted to the impressive variability in oral functioning (particularly in the sucking function) amongst both schizophrenic and non-schizophrenic samples. It is a challenge to our understanding to link up these observed phenomena with personality or organic variables, overt or covert, and it is with this phase of the problem that these investigators have been engaged in recent months, developing and following a variety of leads.

Psychotherapy

Working with a small number of patients in collaboration with Dr. Dittman and others, Dr. Bergman has begun a project on the effect of behavior therapy on central personality disturbances. The plan of the investigation is to determine whether Behavior Therapy, which presumably is effective with neurotic symptoms, can also produce changes in disturbances of central areas of the personality (interpersonal attitudes, sexuality, aggression, self-concept, etc.). In cases where Behavior Therapy does not produce such changes, it may be possible to extend its principles and apply them to these patients.

Human Communication

The study of human communication has been carried on by Dr. Dittmann in the Office of the Chief and by Dr. Boomer in the Section on Personality. The program has focused on specifying the properties of a unit of speech, the phonemic clause, which takes into account the rhythmic characteristics of talking. The phonemic clause is defined by patterns of features such as pitch and stress, and by certain kinds of terminations called junctures which mark the boundaries of clauses.

The hypothesis underlying the research is that the phonemic clause is the fundamental unit into which spontaneous speech is formu-

lated and uttered by the speaker and understood by the listener. Evidence from a study of speech and body movement supports this view. From the speech side, syntactic errors and hesitation phenomena have both been related to the phonemic clause. The relationships with movement are most clearcut in the case of hand movements, less clear, but still definitely present, in the case of head movements. Basically the relationship parallels to some extent that of hesitation phenomena in speech. Foot movements do not appear to be associated with speech rhythms.

The fact that hand and head movements are associated with speech rhythms and foot movements are not opens the way for new hypotheses about the expressive values of all these phenomena. Those movements which are tied to speech patterns may be conceived as more closely related to cognitive processes. Foot movements may be considered to reveal tensions which are less subject to cognitive control, their frequency thus yielding information of a different kind than that of speech patterns. Further experiments in which all of the relevant variables can be included at once are now in the design state.

During the first part of the year Dr. Dittmann was completing his tour of duty at the Gemelli Institute in Milan, Italy. The primary goal of his work there was to participate in the research of the Institute on the psychological effects of the motion picture situation on viewers, with emphasis on viewers' identification with the characters depicted in the film. This identification has been found to be a major hurdle in the use of film records of interviews at NIH in the study of psychotherapy.

The independent variables manipulated in two studies of identification at the Gemelli Institute were frequency of repetition of short sequences of film and length of interval between repetitions. The first study, reported last year, gave equivocal results on the identification process, although it was seen that repetition and a longer interval between repetitions produced greater retention of the cognitive subject matter of the film. The second experiment used a greater number of repetitions and psychophysiological measures of involvement with the film. Here the results were clear that involve-

ment in the film decreased with repetition. There was some indication that a longer interval between repetitions was also effective, though this result was not statistically significant.

CHILD RESEARCH BRANCH

Clinical Investigations

The fiscal year 1965 has been an eventful one for the Child Research Branch. During this year the results of the first longitudinal study emerged for the first time in all three of the areas of research with which the Branch is concerned. The individual project reports should be consulted since they present this work in greater detail. In the work of the Section on Infant Development the finding last year of a male congenital syndrome has been further studied and reviewed for a wide variety of possible errors of interpretation. As Richard Bell's report indicates, the syndrome continues to hold up under scrutiny from a variety of points of view, despite the great difficulties of identifying a firm linkage between a neonatal behavior pattern and a behavior pattern in the nursery school laboratory. This careful re-analysis of data with a view to eliminating a variety of possible artifacts (such as the influence of unreliable assessment of states of arousal in infancy or the influence of family density at age two and a half) provides an increasingly differentiated frame of reference for future conceptual and methodological approaches to other congenital behavior syndromes which may yet be investigated.

Other work of this Section also demands mention. The linkage between autonomous social behavior in two and a half year olds and a tendency toward analytic descriptive perceptual style at age six, even though it has a tie with certain aspects of intelligence, represents an important set of exploratory findings by Frank Pederson and Paul Wender. Although this study requires replication, it is a "first" in the literature to link social behavior in young pre-school children with later cognitive aspects of ego functioning. This is the type of linkage which has been postulated on the basis of clinical experience and psychoanalytic theory since

the publication of Heinz Hartmann's "The Ego and the Problem of Adaptation." In Hartmann's ego psychology the cognitive functioning of the individual lies at the basis of other adaptive styles.

Richard Bell's annual review of developmental psychology has been well received by other investigators in this rapidly expanding field. George Weller's use of sex differences in GSR thresholds in newborns to index differences in the maturity of males and females at birth is an original contribution and provokes speculation about the possibility of better operational definition of the concept of degree or level of maturation in the neonate. Mary Waldrop's and Richard Bell's discovery of an apparent association between family density and congenital behavior styles raises many difficult theoretical questions which challenge current formulations of the interaction of family variables with early personality development; as with much of the work of this Section, this study tends to point up the neglected significance of biological factors in influencing significant behavior developments.

The Section on Family Development has been involved in two different kinds of activities, the data analysis of the husband-wife relationship variables for the first period of marriage prior to pregnancy and the exploration of completely new assessment procedures for studying the marriage relationship. With regard to the former activity, Robert Ryder has processed several thousand bits of information on each couple using repeated and varying statistical and computer approaches, modified by conceptual guidelines. By this means a half dozen stable psychological dimensions of the early marriage relationship have been identified. While these do need to be replicated in a larger study now planned to begin in 1966, these findings represent the most complete and systematic psychological description of average early marriages that exist. Several of these dimensions merit much further study and will serve as foci over the next several years for further research. These include the identification of a type of husband-wife communication in which one spouse distorts his or her report of what is perceived in reality in order to maintain marital harmony. While this phenom-

enon is not at all new in research in ad hoc small groups, the frequency with which it appears in apparently well-adapted families leads one to question past assumptions about the relationship of communication to perception, as one family member accommodates to another.

Over the past year Arden Flint and Paul Blank have become interested in the possibility of observing and judging qualitative differences in moments of shared interpersonal experience of a husband-wife pair. Heretofore the Section on Family Development has focused for the most part on assessments of communication behavior, marital role perceptions, and family values. New techniques of filming are being explored to determine whether access can be gained to aspects of the marriage relationship which are difficult for couples to report upon and also difficult to observe with more traditional psychological techniques.

The Section on Parent-Infant Behavior has carried out initial data analysis of two studies of infants under three months of age in interaction with their parents, using naturalistic observations as well as experimental situations. A variety of interesting sex differences in infant behavior have been identified including the apparent fact that male infants cry more frequently than female infants and that certain mothers tend to rock and hold the male infant more than the female infant, regardless of the amount of crying. In addition, parents tend to spend more time evoking smiles from female infants than from male infants. Whether these findings should be interpreted in relation to congenital sex differences in behavior or to maternal personality variables, or both, remains for future research. Other differences in relatively simple aspects of behavior have also emerged as between males and females which will be followed up in future studies. Also during this past year Howard Moss and Wells Goodrich have begun data gathering on the cross cultural study of eighteen-month-old male firstborn infants and their mothers. Two-hour home observations using a pre-coded time sampling method provide measures of cognitive behavior, autonomy, management of aggression and dependency, verbal behavior and affective responses. On the basis of previous research by Caudill and others, predictions have

been made as to differences anticipated between Japanese mother-infant pairs and American mother-infant pairs and American mother-infant pairs.

The above fragmentary review of work going on in the three Sections of the Branch does serve to suggest variables and hypotheses around which the 1966 longitudinal study may well focus. Now that it is possible to separate husband-wife pairs who employ a highly rational style of communication in problem-solving situations from couples who employ an emotionally-charged mode of communication, it becomes tempting to examine whether these differences correlate with differences in maternal nurturance and control of the young infant and whether these in turn have any influence on the child's emerging social behavior or cognitive style. A mother who avoids fostering dependency in her infant and a family communication context of rationality, one would suppose, might foster the development of an analytic-descriptive cognitive style. On the other hand, if one is willing to extrapolate a little bit from recent findings of the Section on Infant Development, it seems equally possible that autonomous social behavior and analytic-descriptive cognitive style may represent a developmental channel which, in some infants, may be identified in the neonatal period. Such speculations point the way to program directions for the next five to seven years as we continue to investigate developmental patterns and attempt to differentiate between the psychosocial and the biological bases of behavior. They do illustrate some progress in our formulation of research questions since the program of the Branch started in 1959.

ADULT PSYCHIATRY BRANCH

Although the program of the Adult Psychiatry Branch is so diverse that generalizations about it are imprecise, two main clusters of interest in the program can be distinguished. The first cluster mainly concerns psychosocial problems, not exclusively, but in emphasis; the second emphasizes psychobiologic issues. In addition to the research itself, the program includes clinical psychiatric services and treatment which, on the one hand, facilitate research objectives and, on the other hand, can be en-

riched by research concepts and discussion. Also, staff members in the Adult Psychiatry Branch have assumed an increasing responsibility, together with other staff in the Intramural Program, for research and clinical training of psychiatrists.

While the deepening purposes and widening scope of the Branch program have steadily increased its significance and value in recent years, at the same time these developments have brought about the necessity of further differentiation and organization of responsibilities within the administrative structure of the Branch. Administrative shifts in the Branch organization of various clinical, training and administrative functions, now within the Office of the Chief, are therefore being discussed and planned at present.

Research activities are divided into five sections as follows: (1) Family Studies, (2) Studies of Personality Development, (3) Twin and Sibling Studies, (4) Studies in Psychosomatic Medicine, (5) Studies of the Psychophysiology of Sleep.

The clinical work is conducted within two 12-bed wards in the Clinical Center. In addition, when appropriate, the cooperation of hospitals and research organizations elsewhere is obtained in order to gain access to larger samples of research data. For example, we have been able to analyze psychological test data previously collected elsewhere in order to verify leads which have developed from data obtained in our own program. Although most of the research in the program is with human beings, it has become increasingly important in recent years for certain hypotheses to be explored first in animals; improved resources for animal studies constitute an urgent need in the psychosomatic and sleep studies.

Family Studies

Three sections in the Adult Psychiatry Branch, the Family Studies Section under Dr. Wynne, the Section on Personality Development under Dr. Shapiro and Dr. Silber, and the Section on Twin and Sibling Studies under Dr. Pollin, all regard the family as an area of concern, although, as will be indicated, they work with somewhat different kinds of fami-

lies and use different concepts and methods in their work.

In the Family Studies Section there have been five main foci of work: (1) the study of the technique and processes of family therapy and of the conditions and limitations pertinent to its use; (2) the development of systematic research techniques for the direct study of intrafamilial transactions, especially how family members communicate and relate to one another; (3) the study of individual schizophrenic functioning, using methods and concepts which lend themselves to making predictable links with family patterns and which can be used comparatively with nonschizophrenic individuals; (4) the study of individual and family psychopathology from the developmental standpoint; (5) the evaluation of hypotheses linking individual psychopathology and family patterns by studying their applicability in a variety of social class and cultural settings.

Family Therapy

Family and marriage therapy are rapidly becoming recognized as important additions to the traditional psychiatric treatment repertory. An approach especially emphasized in the Family Studies Section and in the Section on Personality Development (Dr. Shapiro) is the technique of having one or two therapists, most commonly a psychiatrist and a psychiatric social worker, meet together with all of the members of a family. This approach has been variously termed conjoint family therapy, family unit therapy, or family group therapy. The immediate, on-going transactions of family members with one another and with the therapist are regarded as the most significant starting-point data to be explored, understood and treated in this approach. It has become increasingly apparent, once that therapists have broadened their vision to consider more than an individual presenting patient, that many psychiatric problems cannot appropriately be located within individual family members but are problems of communication and relationships that often involve all members of a family or household.

In other instances, it may not be practical or appropriate to treat the family as a unit to-

gether on an intensive, exploratory basis, but nevertheless a treatment program that includes more contact with the family than traditionally occurs may facilitate or even make possible the treatment of individual family members. It has been frequently observed that hospitalized and non-hospitalized schizophrenics are frequently taken out of treatment just at the point where the therapist sees some change beginning to take place in the individual patient. With conjoint family therapy, or sometimes with other kinds of collaborative contacts with the other family members, such disruptive failures of understanding of the treatment by other family members may be forestalled and, in some cases, the family may actively be able to support the treatment effort.

A special difficulty in psychotherapeutic work with families is that the possible arrangements under which the therapy can be set up are much more varied than in individual psychotherapy. A good deal of clinical research will be necessary to evaluate the usefulness of alternative approaches and arrangements and also to consider methods of dealing with treatment impasses. For example, the problem arises of who should be included in the family therapy group when there are young children in the family, or when there are persons in the household who disclaim interest or personal involvement, or when there are persons outside of the biological family who do seem involved in the intrafamilial problem. Another issue deserving study is how therapists can best deal with family members who either are non-participant or are constantly disruptive of the participation of others. Also, a good deal more work needs to be done on the training of family therapists to deal with the multiplicity of emotional stresses that occur in the conjoint family therapy situation. These are only a few of the kinds of issues that need extended evaluation before the conditions under which family therapy is indicated will be understood. A clinical paper has been published this year by Dr. Wynne in which he assesses some of these problems.

Quite clearly, only a relatively small number of variations in approach can be used by any treatment staff at a given time, so it is especially important that different approaches and

the conditions under which they are used be discussed and evaluated. Unfortunately, the demand for family therapy far exceeds its availability at present and many clinics where it is now being carried out are under such pressure to provide services that they are unable to take an evaluative approach to these questions. In the Adult Psychiatry Branch we plan that our activities along this line will be expanded and developed during the next few years.

A special and growing interest in family therapy and family psychiatry arises because it can be regarded as a foothold or starting point for the participation of clinical psychiatrists in broader comprehensive treatment programs and preventive psychiatry. Not only is the family the most immediate and most emotionally significant interpersonal influence in the psychological development and functioning of most individuals, but it also constitutes the most accessible naturally occurring group in society with whom psychiatrists can use the skills developed in ordinary clinical training. Although a good many psychiatrists are now developing an interest in broader comprehensive treatment programs and in community and social psychiatry, work with community groups, agencies and larger social organizations ordinarily involves indirect, rather than immediate, contact with persons who are psychiatrically suffering. It therefore is less personally satisfying and makes less use of previous training than many psychiatrists would prefer, especially if the wish to carry out therapeutic work with troubled persons has been a primary motivation leading to the choice of a career in psychiatry. Thus, family psychiatry may serve as a bridge between traditional, individually-oriented psychiatric programs and broader social psychiatric programs of the future.

In addition to the study of family therapy as a treatment technique in its own right, the use of family therapy has served two other research purposes: it has provided a relatively unstructured, free-wheeling source of research hypotheses and unanticipated ideas, and tape-recordings of the psychotherapeutic interviews with families have been extracted for special research purposes.

Studies of Intrafamilial Communication and Transactions

During recent years a considerable number of specific findings have been documented in the empirical research of the Family Studies program. This work has shown that the kind of psychopathology found in an individual offspring can be systematically and accurately linked with the style and form in which family members communicate and relate to one another (that is, have transactions with one another). Five papers have been published or accepted for publication on this research during the past year, and a number of other reports are being prepared for publication. Here only a few features of this work can be mentioned.

a. Several varieties of schizophrenics and diverse neurotic patients have been distinguished from one another "blindly" by several judges who were given data only from other members of the families of these patients, *not* from the identified or presenting patients themselves.

b. These "blind" differentiations have been accomplished using diverse kinds of psychological data ranging from (1) individual projective tests; (2) conjoint family test procedures in which no interviewer or tester is present in the room, but the transactions are tape recorded (Spouse and Family Rorschach); (3) clinical interviews with family members; and (4) a test of "thinking disorder" (Object Sorting Test). Significantly, the predictions about the offspring from family data of these various kinds have all made use of similar criteria. These criteria or guidelines emphasized the *style* and *form* of the communication of the family members rather than the *content* of thinking, which appears to be a relatively inferior basis for predictive differentiations. By *form* of communication is meant *how* attention is focused and organized (rather than *what* the content is); whether attention is maintained with continuity appropriate to the situation or task, or whether attention drifts off vaguely or abruptly and confusingly shifts; whether attention is fixated at excessively abstract, over-inclusive levels or at under-inclusive, concrete levels, or shifts erratically from one level to another. These and other criteria have now been

spelled out and illustrated in recent publications. They apply to individual styles of handling attention and, even more importantly, to how family members *share* foci of attention in their communication (transactions) with one another.

c. Although considerable intuitive and integrative skill was originally necessary on the part of the judges who have interpreted and differentiated the family data, efforts have been made to make these differentiations a matter which can be carried out by others by using certain scoring and judging rules. During the past year work has progressed with scoring manuals for use with the individual Rorschach, the Thematic Apperception Test and the Object Sorting Test (Dr. Margaret Thaler Singer), and for the Spouse and Family Rorschach procedures (Dr. Nathene Loveland). Data using these manuals have now been applied to various subgroups of families within the total of 250 families from whom psychological test data is now available. Reliability of the scoring procedures seems high but is being evaluated further, and a series of preliminary findings using the individual Rorschach scoring manual have been reported.

d. It needs to be stressed that the blind differentiations made from family data about patient-offspring include but go beyond gross discriminations such as the global diagnosis of schizophrenia, psychoneurosis, or "normality." It is now possible to make these predictions with a high degree of specificity and accuracy along three dimensions: form of thinking (four varieties), degree of severity of psychotic tendency (five levels), and variety of affect disorder (five levels).

e. Throughout this research use has been made of the concept of the family as an organization of roles and role-expectations. Using these and related formulations about the family as a social and psychological unit, Dr. Singer has been able to match patients with their families blindly on the basis of projective test protocols. A "matching study" with 35 families showed statistically significant results and has been published this year. As methods of directly studying intrafamilial transactions, the Family and Spouse Rorschach techniques have received special attention during the past

year. They appear to provide data for systematically analyzing very important issues about intrafamilial transactions. Briefly, the procedure is as follows. After family members have received the individual Rorschach test in the usual fashion, they meet together as a marital couple or as a family unit. After receiving standardized instructions, they discuss and interpret the Rorschach cards with each other and attempt to reach a consensus without an examiner present during the discussion which is tape recorded and observed through a one-way mirror. This procedure has now been administered to some 70 families, ranging from families with severely schizophrenic offspring to borderline, neurotic and normal families with and without medically ill offspring. This procedure seems sufficiently interesting and unstructured to elicit from families a rich sample of their modes of communication in their own terms; at the same time the procedure is sufficiently standardized so that characteristic transactional patterns can be differentiated and scored for a wide variety of families. As in the other studies in this program, the style or form of how they go about the task, especially how they share, or fail to share foci of attention, and how they reach, or fail to reach, closure and consensus constitute the primary data of importance, rather than the content of responses as such.

The Rorschach technique has thus been modified to allow comparison of behavior in a variety of constellations: the transactions of each family member with a tester, the parents with one another (in the Spouse Rorschach), the parents with "well" offspring (patient absent), the parents with the psychiatrically disturbed offspring (well sibling absent), and all the family members with each other. This and other techniques of studying family transactions in standardized situations, such as the Revealed Differences technique, which has also been used in the Family Studies Section, provide new and valuable methodologies for testing hypotheses about families. These and related approaches will be a major focus of research activity in future programming in this Section.

In another study of parental forms of communication, Dr. Gary Morris and Dr. Wynne have published their findings this year in

which excerpts from tape-recorded family therapy interviews were examined by a judge who had no diagnostically relevant data about the offspring. Excerpts from conjoint family therapy with 12 families were studied; the index offspring in these families had a variety of schizophrenic and neurotic illnesses. Predictions about the offspring from the parental excerpts were found to be highly accurate along all of four dimensions—over-all psychiatric diagnosis, form of thinking, severity of psychosis and kind of affect disorder—when compared with independent clinical ratings of the offspring.

Still another method of stimulating and evaluating intrafamilial transactions which has been developed in the Adult Psychiatry Branch is family art therapy. In this approach, art media are used to facilitate expression and communication in families seen together by the art therapist, Mrs. Hanna Kwiatkowska, and a cooperating investigator (psychiatrist or social worker) as a participant observer. The analysis of the art productions of the session and of the recordings of the session with these families has shown striking similarities in the style of thinking and perceiving of different family members. The initial work with families where one of the members is afflicted by a schizophrenic illness brought valuable observations on thought disorder in schizophrenics and led to further work with families where one member was afflicted with other kinds of different mental disorder.

Beginning in 1964, all the families studied by the Family Studies Section and by the Twin and Sibling Section, most of the families of the Section on Personality Development, as well as other normal control families, have been seen in art therapy evaluation. The family art therapy evaluation session is considered as part of the over-all family evaluation procedures and is ordinarily used with newly admitted families in the first two weeks of their admission.

The art therapy evaluation session is structured in such a way that it combines spontaneous self expression with standardized procedures for comparison of different families. In this single evaluation session the dynamics of the family, the splits and alliances, the pairing

off, the delineation of roles, the impact of parental interaction on the offspring are impressively displayed and provide an unusual field for observation often not obtainable as rapidly in purely verbal action. The art productions themselves as well as family discussion of this material provide interesting data for study.

Later it is decided if the family will be seen as a group in long-term family art therapy, or if the patient will be seen in individual art therapy as an adjunct to family and individual psychotherapy.

Individual Schizophrenic Functioning

Work has continued during the past year on the development of an improved classification of schizophrenic disorders, based upon and extending Bleuler's original formulations of the schizophrenias. Dimensions of functioning have been defined along which schizophrenics and other individuals can be rated both from clinical study and from a variety of psychological tests. Previously, a new classification of schizophrenic forms of thinking was published. This year, and next, work is being done on a refinement and extension of this classification, with the use of a new series of rating scales and test procedures. A series of continua in addition to the dimension of styles of focusing attention and thought are being considered. These deal with energy level and affective disorder, forms of interpersonal relatedness, and disturbances of the self-nonself ("ego") boundaries. Also, scales dealing with the premorbid development history of schizophrenics and the kind of onset of the illness are being modified from scales developed previously elsewhere. The available scales have been largely applicable to only portions of the patient population (such as male veterans) and have been poorly conceptualized because of the inclusion of particular etiologic biases rather than keeping the scales on a descriptive basis. Dr. Julian Silverman who will join the Intramural staff in July 1965, has been working on a contract basis this year on a number of important studies in which some of these dimensions are studied with a series of new tests of attentional and cognitive processes applied to differing subclasses of schizophrenics.

These studies of schizophrenic psychological functioning are designed to facilitate the further evaluation of predictive links between individual patients and family patterns by studying all the family members. Most studies of families of schizophrenics in the past have had little more than historical and attitudinal data about family members other than the presenting patient. This research endeavors to obtain much more detailed material on the attentional and thinking processes of all the family members, as well as the direct transactional data described above. In addition, further delineation of continua relevant to schizophrenia should contribute to the improved understanding of schizophrenic functioning as a research question in its own right.

Developmental Aspects of Family Studies

The blind differentiations described above of families with different kinds of offspring have all made use of a series of developmental hypotheses in which it is assumed that the individual's biologic capacities for focusing attention and for perceiving, thinking, and communicating gradually are shaped and modified by interchange with the environment during development. This viewpoint is epigenetic: the interchanges or transactions at each developmental phase built upon the outcome of earlier transactions. This means that constitutional and experiential influences recombine at each developmental phase to create new biologic and behavioral potentialities which then help determine the next phase. If transactions at any given developmental phase are distorted or omitted, all the subsequent developmental phases will be altered because they build upon a different substrate. This research has utilized the hypothesis that parents and their offspring fit together in a particular family constellation which changes over time both for the family members as individuals and for the family as a social unit. What may have psychopathologic consequences thus varies over time but makes possible inferences about the developmental phases in which particular forms of relating and communicating might be especially important in a child's psychological growth.

These formulations have been important in working out criteria for distinguishing families of schizophrenics who became ill early in childhood, compared to those who became ill later in childhood and late in adolescence or young adulthood. Singer and Wynne have previously shown that the parent-child relationships of autistic children are distinctively different from those of young schizophrenics. During the past year, data has accumulated which indicates in increased detail how the parents of schizophrenics whose illness dates from late childhood and early adolescence differ from those who do not develop significant difficulties until late adolescence or adulthood. This work relates to the differentiation of families of "amorphous" versus "fragmented" schizophrenics.

In addition, a project being conducted by Dr. Franz and Miss Hoover in this Section attempts to focus with retrospective interview data on those aspects of child development and experience which seem to have led one offspring in the family to develop a schizophrenic illness and another to be spared. The study attempts to describe the possibilities for a range of interpersonal influences between the parents and the developing child, not only those which may seem to be pathological. The parents are interviewed concerning the earliest, presumably innate differences between their offspring, and each member of the family is interviewed in an attempt to define the identity, roles, and capacities of each family member. Also, an effort is made to evaluate the ties of each offspring to the parental home, the patterns of emotional distance or closeness within the family, and the patterns of friendship outside of the family in the past, present, and projected future.

Prospective longitudinal, developmental studies are important in the long-range planning of the family Studies Section. They will be initiated when current methodologic and other findings make this timely.

Cross-Cultural Family Studies

The cross-cultural validity of findings on families in the United States is currently being examined through anthropologic and psychological test study of families in Lebanon and in

Japan. Dr. Wynne is currently analyzing a considerable mass of data which were obtained during his field work in Lebanon last year and by his research collaborators, Drs. Herant Katchadourian and Professor Herbert and Mrs. Judith Williams who have continued the data collection during most of the current year. Dr. Wynne has presented a paper on the evaluation of hypotheses about the nature of the "psychological boundaries" around the families of schizophrenics in this country, in comparison with the nature of family boundaries in normal, large, extended families observed in a traditional Muslim village in Lebanon.

Section on Personality Development

The work in the Section on Personality Development has been concerned with clarification of psychological issues pertinent to adolescence and has been carried in two research groups, one under Dr. Roger Shapiro and the other under Dr. Earle Silber.

Dr. Shapiro has continued his work in the study of the identity problems of a group of late adolescents who decompensate emotionally in the first prolonged separation from their parents. He approaches the study of the identity problems of the adolescents through the analysis of parent-adolescent interaction in family therapy sessions. A major focus has been that of the parental delineation of the adolescent in these group sessions in order to obtain data which clarifies the adolescent identity problem with particular emphasis upon separation difficulties.

By delineation is meant the view or image one person has of the other person, as it is revealed explicitly or implicitly in the behavior of the one person with the other person. The research group is currently analyzing the material from 6 families to define the relationship between recurrent delineation of the adolescent by the parent's and the adolescent's identity problems which are formulated from research interviews and psychological testing.

A central hypothesis of this study is that there is a significant relationship between parental delineation of the adolescent and adolescent identity formation. Further, it is hypothesized that in families in which the adolescent is

seriously disturbed psychologically, there will be evidence of striking distortions, inconsistencies and contradictions in parental delineation of the adolescent. The study of the parent-adolescent relationship includes questions about the kinds of parents who are particularly reactive to various manifestations of adolescent development; the manner in which these reactions, or vulnerabilities, are expressed; and the effects of these parental behaviors on the personality functioning of the adolescent.

A highly important subgroup of delineations consists of those which we call defensive delineations. When behavior of an individual with another person contains evidence of distortion of the other person in terms of the individual's own defensive organization, then defensive delineation exists. Parental delineation of the adolescent is assessed with the hypothesis that parental defensive structure will be an important determinant of their delineation of the adolescent. Research interviews are conducted with the parents with the goal of elucidating important areas of parental conflict and their characteristic defensive operations. The research group arrives at an understanding of interactions in the family session by clarifying the relation of parental defense to characteristic parental delineations of the adolescent.

The following method is employed in the analysis of family interaction for delineation. Excerpts of interaction are isolated in a series of family sessions which are characteristic of the parent-adolescent relationships. In the analysis of these samples of family interaction containing delineation, the following questions are asked:

(1) What is the reality situation in which these interactions occur. What is the nature of the actual events and issues with which the family members are dealing; and what is the adolescent's behavior in the reality context under discussion.

(2) What delineations of the adolescent by each parent can be inferred from this particular interaction.

(3) Is there an apparent distortion in the parent's view of the adolescent. This may be a clear and obvious disjunction between the view of the adolescent expressed by the parent and

what is seen by the observer. It may become evident only when the delineation is viewed in the context of a particular reality situation and specific behavior in reality of the adolescent. It may become evident from contradiction or bias expressed in delineation. The parent's inappropriate, biased, exaggerated or idiosyncratic view of the adolescent can be defined by an observer. If the delineations contain evidence of distortion, then the inference of defensive delineation is made.

(4) What hypotheses about the determinants and structure of defensive delineations can be formulated. These inferences are made clinically from the material of the family session. They are compared to formulations from research interviews and psychological tests of the parents which define areas of conflict and need, and organization of defenses. If there is good correspondence between the dynamics of defensive delineation and evidence from the individual assessment about individual psychodynamics, then it is assumed that the delineation is a manifestation of relatively enduring parental character structure. This supports the argument that defensive delineation is not simply a reflection of the adolescent's actual characteristics, but represents an expression of the parent's needs and defensive organization. These lead to characteristic emphases and distortions of delineation of the adolescent.

In assessing the impact of parental delineation upon adolescent personality development, thought is explicitly given to the kinds of personality organization in the adolescent which are particularly vulnerable to delineation.

Dr. Winfield Scott, the psychologist in this research group, has been analyzing the projective tests of the parents and of the adolescent with these questions in mind. He is matching adolescents to sets of parents using a technique developed in consultation with Dr. Margaret Singer. One of the characteristics of adolescent and parent which was helpful in four successful matchings done recently was the inference of degree of dependency upon external stimulus required by parent and adolescent in order for them to find meaning in the projective test situation. In one family there was evidence of great inner uncertainty in both parent and adolescent, with weak inner determinants

of percepts, great confusion about the meaning of anything, and great dependence upon the external stimulus to provide meaning.

This was in great contrast to the material from another family where the imposition of a particular organization of internal difficulty upon the projective material was striking. Here the parent and the adolescent imposed explicit highly idiosyncratic meaning upon an affectively charged external stimulus rather than revealing a search for meaning. In another adolescent there was evidence in the projective tests that he would avoid being influenced by any external stimulus in coming to a conclusion about himself and about what he is like.

These findings from projective tests suggest a variety of formulations about ego functioning in parents and in their adolescent offspring which will allow more precise thinking about both the vulnerability to, and the dynamics of delineation, the meaning of such behavior in the psychology of the parent who is delineating, and the meaning of this behavior for the personality functioning of the adolescent who is delineated.

In the second part of the Section on Personality Development, Dr. Silber and Dr. Tippett have been involved in developing other methods by which some facets of identity problems in the late adolescent period can be studied more systematically. During the past year they have completed a monograph, now in press, which deals with the problem of clinical assessment and measurement validation of self-esteem in late adolescence. In their work, they reviewed psychoanalytic concepts which are related to the construct, self-esteem. Different measures of self-esteem reflecting the definition of self-esteem in the psychoanalytic literature were studied in a sample including normal and disturbed adolescent subjects.

Three particular test measures of self-esteem were used. One measuring instrument was based on a modification of the Role Repertory Test in which the subject develops his own dimensions on which he subsequently rates himself. The other instruments included a self-image questionnaire and a scale for measuring self-esteem used by Rosenberg in his studies of adolescents.

In addition, a method for assessing self-esteem based on clinical interviewing was developed and is described in the monograph with criterion examples for the various types of ratings. The problem of defensiveness is also considered in their rating scheme. Five syndromes of self-esteem emerged from the clinical study of their subjects: Defensive High Self-esteem, Nondefensive High Self-esteem, Inconsistent Self-esteem, Ineffective Defensive Self-esteem, and Low Self-esteem. These clinical groups can be distinguished by the test measures and there are statistically significant differences on the test performance between these groups.

Each of the three test measures of self-esteem met certain criteria of validity: (1) each was more highly correlated with each of the other measures of self-esteem than with another concept which was measured by the same method; and (2) each of the measures of self-esteem was also more highly correlated with each of the other self-esteem measures than with a measure of another concept assessed by a different method. The work also reveals that many of the subjects who scored differently on different test measures comprise a distinct clinical group characterized by inconsistent self-esteem.

By first carefully establishing the evidence for the validity of methods for measuring self-esteem, the work opens up the possibility of studying self-esteem in relation to other aspects of personality functioning.

A second phase of their research involves an experimental procedure for studying the autonomy of self-esteem, specifically, the extent to which self-esteem can be altered by authoritative suggestion. An experimental group was given fictitious authoritative ratings about themselves prior to their making a new rating of themselves. The influence of this procedure on the subsequent reporting by the subject about himself was compared with a baseline measure of the subject's self-ratings and with a control group. Reporting about the self-image was altered to a significantly greater degree for the experimental subjects when compared with the control group. The relationship of other personality variables to change resulting from this procedure is being studied. At the present time,

this facet of their research is nearing completion with the preparation of a paper describing the experimental study. The methods used offer techniques for studying one of the special characteristics of late adolescence, namely, the potential of an outside authoritative source for influencing the adolescent's own self-image.

During the current year, the focus of the research is shifting to exploring the relationship between self-esteem and other ego functions: particularly the defensive, adaptive and perceptual function. In collaboration with Dr. Holt, Rorschach protocols have now been scored independently in accordance with Holt's criteria for assessing primary and secondary process thinking and various measures of defense. These and other personality variables will be related to the measures of self-esteem.

Section on Twin and Sibling Studies

During the past several years, Dr. William Pollin, Dr. James Stabenau and the staff of the Twin and Sibling Section has concentrated upon the comparison of discordant siblings or twins within a given family as a technique for the study of schizophrenia and, more generally, personality formation. Emphasis has been placed upon the nuclear family group comprised of both parents and two or more offspring who are discordant for schizophrenia. In one study the offspring are twins; in another, they are ordinary non-twin siblings. Matched control families free from psychopathology, or with severe non-schizophrenic psychopathology, have been similarly studied.

Findings to date are relevant to three significant questions: (1) Are there any distinguishing features which differentiate the life history of a schizophrenic from a nonschizophrenic individual within a given family; (2) are there any distinguishing features which differentiate families in which schizophrenia develops from other families within which no psychopathology or other types of psychopathology have developed; and (3) are there any characteristic features that the schizophrenic patient-to-be presented in his pre-illness childhood.

During the past year, the series of identical twins discordant for schizophrenia was enlarged from five to eleven, and a beginning was

made upon the study of matched control twins and their families. The first 12 families admitted in the twin series were reviewed with respect to psychiatric data, and findings relevant to the question of life history differences between identical twins discordant for schizophrenia were presented. Analysis of the data obtained in the study of siblings discordant for schizophrenia, and matched groups of control and delinquent families, continued, with presentation (1) of the findings derived from an interpersonal rating system; and (2) of a new technique for analyzing the TAT protocols which made possible the blind identification of normal, delinquent and schizophrenic families on the basis of different patterns of parent-child interaction. Papers were published (1) describing the first five families in the Twin Study and (2) distinguishing characteristics between schizophrenic, delinquent and normal families from the Sibling Study. Conflict resolution studies were limited during this year to further filming of family conflict resolution sessions, employing the Revealed Differences technique described in previous reports.

In previous years a total of 26 families comprising 104 individual subjects have been evaluated in the Sibling Study. The study protocol included group and individual psychiatric interviews, Rorschach, TAT, Object Sorting Test, Leary Interpersonal Check List, Revealed Differences, Color-Matching Test, Gottschalk Interview, Embedded Figures Test, Q-Sort Moral Value Scale, Cornell Medical Index, MMPI, Parental Attitude Research Index and Child Attitude Research Index. Each family was seen in a home visit and a Goldfarb rating scale was completed.

The Twin Study is an outgrowth of the Sibling Study, using the same core methodology, in expanded form, and with relevant additions. Additionally, 11 collaborative investigators have undertaken simultaneous evaluation of the twin families employing 39 different psychiatric, psychological, biomedical, physiological and medical investigative procedures. Thus far a total of 15 families have been admitted to the Clinical Center for Twin Study evaluation, of which 13 currently constitute the ongoing series. (The first family was for trial-run pur-

poses; clinical complications during the stay of Family #13 prevented completion of the work with them and necessitated their being dropped from the series.) These include to date two normal control families, four "borderline" families and seven in which the index twin is clearly schizophrenic.

Major results to date include the following: (1) A pattern of consistent life history differences has been found to differentiate the schizophrenic from the nonschizophrenic twin in the series thus far studied here at NIH. Prominent features of this pattern include the following: (a) the schizophrenic twin-to-be has in each case weighed less at birth, and as a group have demonstrated more "soft" neurological signs than the co-twin control; (b) tended in childhood and adolescence to be less competent, organized and effective than was his co-twin, and (c) tended to be the more sensitive, anxious and unhappy of the twins from a very early age on. The data, though consistent thus far, are based on too small a series to permit extensive conclusions to be drawn; as yet, it is not clear as to whether they relate to schizophrenia *per se* or, more generally, to a susceptibility to various forms of psychopathology. Our current, tentative formulation of these data is that in the group of families studied, initial, non-genetic, constitutional differences contributed to or determined the very early establishment of role differences within the family relationship pattern. The smaller twin, as a result of these relationship and role differences, experienced a sequence of reinforcing events in childhood years which *in toto* accentuated rather than mitigated the initial minimal disparity in coping potential. The increasing intertwin differences in personality, particularly in ego structure, led to an increasingly unfavorable stress-coping ratio, in the index twin, with passing years. That is to say, there was an increasing tendency to generate stress in dealing with ongoing developmental events and transitions, and a relatively decreased ability to cope with them.

(2) Biochemical studies: Three collaborative studies have been under way with extramural investigators whose reports of positive findings in schizophrenia have occasioned considerable

interest in the past several years: (a) Dr. Arnold Friedhoff, Department of Psychiatry, New York University, New York City, who reported finding a new catecholamine metabolite in the urine of schizophrenic patients and not in the urine of normal controls; (b) Dr. W. J. Fessel, Langley Porter Psychiatric Institute, San Francisco, California, who described serum differences (S 19 macroglobulins) and abnormal blood cells in schizophrenics compared to normals; and (c) Dr. Charles Frohman, Lafayette Clinic, Detroit, Michigan, who has described a possible plasma factor related to schizophrenia, on the basis of abnormal lactate/pyruvate ratios. Each of these investigators has been running blind analyses of the appropriate samples drawn from both twins, and in some instances from the parents and additional patients and controls, as well. Dr. Friedhoff has thus far not analyzed a sufficient number of sample to permit any conclusions to be drawn. The results obtained thus far by Dr. Fessel have not confirmed previous reports; however, the series is thus far not complete with regard to abnormal blood cells. Dr. Frohman has been successful to a considerable extent in correctly distinguishing the schizophrenic from the nonschizophrenic twins. His procedure involves blind judgment as to whether any given plasma sample has been taken from a schizophrenic or nonschizophrenic individual. The judgments are based on measurement of the lactate/pyruvate ratio. Of the 9 pairs studied, 8 of the 9 schizophrenic index twins were correctly called schizophrenic on one or the other of their two samples; 13 of their 18 samples were so identified. Conversely, of the 8 control twins from the same pairs (one control has not yet been tested by this technique), one was called schizophrenic and one questionably schizophrenic; two of the 16 samples were judged to be schizophrenic or possibly schizophrenic. These differences are statistically significant at the .01 level. (Of the total of 169 NIH samples thus far analyzed by Dr. Frohman in this study, including parents and non-twin controls, 81 of 101 normal samples and 34 of 68 schizophrenic samples were correctly identified.) These findings are statistically significant ($P > .001$), but show a substantial number of false negatives

and positives. Six of the 9 twin fathers gave such positives, and only one of the 9 mothers; none of these parents were clinically schizophrenic. These findings are of considerable interest, and though their relationship to schizophrenia is not as yet clear, they clearly call for further work along these same lines.

Whereas ongoing current analysis of Twin Study data at the moment has been focused upon the psychiatric and life history material and upon the preliminary biochemical data, analysis of the Sibling data thus far has concentrated upon the structured psychological and interpersonal tasks which were part of the study protocol. A report published this year described the findings in a carefully cross-matched sub-sample of five normal, delinquent and schizophrenic families. Characteristic patterns of familial interaction were found which differentiated the three groups of families. These differentiating modal patterns were separately revealed and confirmed by blind judgment of the Thematic Apperception Test data and by objective analysis of recorded and filmed sessions of the Revealed Differences technique, and were consistent with prior clinical judgments and hypotheses. The significant differentiating characteristics included variations in stability and reliability of the intrafamilial relationships and the execution of family roles; quality and mode of expression of affect; parental perceptions of children's roles, potentials and corresponding modes of control; and degree of personal autonomy and differentiation permitted or encouraged. Additional differences between these families were found in the area of conceptual thinking, as demonstrated by objective assessment and blind ratings of the Object Sorting Test, and in levels of communication clarity versus impairment. Thought disorder was found in the parents of both the schizophrenic and the juvenile delinquent indexes and similarly communication pathology and impairment were found in both of these groups of families.

An analysis of family interpersonal, self and ideal perceptions obtained by use of the Interpersonal Check List was presented. This procedure revealed further distinguishing features differentiating the three groups of families.

These included the finding that the mothers of the schizophrenic indexes were experienced as more hostile and dominant by other family members, and also rated themselves as more hostile and dominant than the normal mothers did. However, it was noteworthy that the same pattern, to a considerable extent, characterized the mothers of the delinquents as well. This pattern of increased maternal domination/hostility has often been related to schizophrenia, but in these families related to psychopathology rather than being specific for schizophrenia. Another finding of interest in these data was that when retrospectively rated for pre-illness personality, the schizophrenic indexes were seen by family members as being more submissive and less dominant than their non-schizophrenic controls. This is in keeping with the picture of life history differences obtained from the families of the discordant twin series.

The present program of the Section calls for continuing expansion of this series of twin families. We hope to increase both the number of pairs discordant for schizophrenia, and of matched normal controls, and additionally to introduce a matched control series of twins discordant for other types of severe but nonschizophrenic psychopathology. Another major planned addition is a program of extended home and community visits which is now beginning. The Sibling Study, on the other hand, is not at the current time programmed for further expansion of its subject sample. Such expansion may occur at some time in the future following more complete analysis of the Sibling Study and the Twin Study data, as a means of testing conclusions and hypotheses derived from such analysis.

The differences described above between schizophrenic and nonschizophrenic twins and siblings are consistent with a number of papers presented by other investigators during this past year which attempted by other means to get at the same question—how did the schizophrenic before the onset of the disease differ from other members in his sibship who did not become schizophrenic. These several reports agree in picturing the schizophrenic in childhood as being less competent and well organized, having a lower I.Q. and a higher level of

psychological discomfort and non-specific difficulty. These apparently convergent reports raise the hope that we are making significant progress in defining the pre-psychotic patterns and characteristics of schizophrenics-to-be, and may thereby contribute to possibilities for future prophylactic measures with respect to this major psychiatric illness.

Section on Psychosomatic Medicine

The work of the Section on Psychosomatic Medicine is concerned with interrelationships between biology and behavior. Specific projects range from laboratory research at the molecular level to molar but detailed clinical investigations. The overriding and unifying theme of the Section's work is the furtherance of a meaningful understanding of the behavioral-biochemical paradigm. Individual projects may be summarized as follows.

There is a good deal of inferential evidence from both clinical and animal studies that norepinephrine (NE) metabolism in brain may markedly influence a variety of behaviors. However, due to several methodological problems direct estimates of norepinephrine metabolism in brain *in vivo* have not been obtainable. Because of the importance of such knowledge for future clinical and animal investigations, much of the Section's efforts over the past two years have been devoted to the development of a technique whereby reliable, individual measures of norepinephrine metabolism in brain may be obtained. In the initial phases of this work it was found that (a) norepinephrine given into the cisterna magna rapidly enters brain and by five hours is in a pool which is not freely accessible to the CSF'; (b) 50–70% of the H³NE enters blood from CSF'; but (c) when rather large amounts of H³NE are injected into blood essentially no radioactivity is found in brain or the CSF. These findings suggested that by the use of the double-isotope technique appropriate kinetic and mathematical models could be derived from urinary assays of metabolic products by which direct estimates could be obtained of (a) the amount of NE which enters brain; and (b) how much and at what rate NE in brain is changed to various metabolites. Experimental work with dogs has indicated that this can be

done reliably. Present plans are to begin clinical investigations using this technique.

Other studies of NE metabolism at a more molecular level are also being done by our group, viz. the enzymatic steps involved in synthesis and breakdown of norepinephrine are fairly well understood but the mechanisms by which the catecholamines may be bound or stored intracellularly in brain are less clear. Because of the structure of the NE molecule a reasonable hypothesis is that coordination with metals may be intimately connected with the phenomenon of binding. In support of this hypothesis we have found that synaptic vesicles isolated from rat brain have more than enough metal (Mg, Cu, Fe, Zn) to coordinate with the amounts of catecholamines present in this subcellular fraction of brain. In addition, ethylenediamine prevents uptake of exogenous NE by vesicles. Lastly, *in vitro* evidence for the formation of ternary complexes between metal, NE, and ATP has been obtained. These findings as well as data reported by a variety of investigators are consistent with, and supportive of, the hypothesis that coordination between metals and NE are importantly related to the intracellular binding of NE. Further work in this area is in progress. In the process of the above investigations it became apparent that metals might also be quite important for membrane function. Data obtained indicate that (a) metals coordinate with phospholipids and alter their solubility characteristics; (b) ternary complex formation between metal, phospholipids, and ATP occurs; and (c) the solubility characteristics of this ternary complex is similar to that of the phospholipid alone. From this data a molecular model of the membrane which can explain reversible transitions between two phases having different physical properties was constructed. This model is such that it can be experimentally tested in biological preparations. This work is now in progress.

There is a good deal of experimental evidence from a variety of animal studies which indicates that during development there are critical periods during which environmental influences are most important in determining the ability of the adult animal to respond to stress, to learn, and in the case of dogs to form social

bonds. In an earlier investigation of some of the biological processes which might underlie this latter phenomenon it was found that during the developmental period critical for primary social formation in dogs there were marked fluctuations in the norepinephrine and serotonin levels as well as catechol-O-methyl transferase action in several areas of the brain. Around the beginning of this critical period amine levels and enzyme activity were high but markedly decreased over the following two weeks. As a beginning in the understanding of the possible significance of this finding for behavior, collaborative work with investigators at the Jackson Memorial Laboratories at Bar Harbor, Maine was undertaken in which one group of pups at 5 weeks of age and another at 7 weeks were given reserpine for a period of 10 weeks, taken off the drug and then tested in a variety of ways at 26 and 36 weeks of age. Control animals were given nembutal. Preliminary data indicate that those animals receiving reserpine at 5 weeks of age are behaviorally different in several respects from both control dogs and those who were begun on reserpine at 7 weeks. Due to the small sample size, firm statements must await the outcome of studies now in progress which have as their goal the replication and extension of the earlier findings. In addition to this work with the dog we have begun a series of investigations with the rat as to the effects of early stress on adult behavior and brain amines.

In a somewhat different approach to the relationship of central nervous system amines and behavior we are continuing investigations of neurochemical-behavioral correlation in strains of mice and rats which have been inbred for differences in *emotionality* or fearfulness as measured by the open-field test. Initially in working with mice which differed in terms of this behavior, it was found that the more "emotional" strain of mouse (BALB/cJ) had a higher level of serotonin when compared with another less emotional strain (C57BL/10cJ). In addition, the work seemed to indicate that the serotonin differential was due to brain stem and limbic structures rather than other areas. Since this initial finding, work has proceeded along lines which attempt

to relate this serotonin difference to behavior in other strains and species. It has been found that (a) in three other strains of mice, one of whom was "emotional" and the other two not, serotonin differences in the predicted direction were found; and (b) reactive and nonreactive strains of rats which were bred by Broadhurst for differences in open-field behavior also show brain serotonin differences in the predicted direction. Correlations between ambulation scores in the open-field and serotonin levels in limbic structures in brain were done and there was found to be a significant negative correlation. Present work in progress deals with obtaining F_1 and F_2 animals from the reactive and nonreactive strains for behavioral testing and serotonin assays.

At the clinical level Dr. Bunney and co-workers have continued their work with biochemical and behavioral aspects of depressive reactions. The basic design of the project involves the development of a research metabolic ward and the construction of a biochemical and behavioral data collection system. A continuous longitudinal method has been developed for obtaining clinical data through the use of rating scales. These scales have proved useful, reliable, and adaptable to a variety of research requirements. The nursing research team has continued to work at an exceptionally high level of performance in the collection of this data and have recently received a superior performance achievement award from the Department of Health, Education and Welfare for their efforts.

A number of present research projects have been stimulated by previous findings which can be summarized briefly as follows: (1) 17-hydroxycorticosteroids (17-OHCS), an index of anterior pituitary-adrenal cortical activity, are elevated in depressed patients; (2) we have found that these high levels exist, however, in only certain subgroups of depressed patients; (3) we have continued to confirm our findings that high positive correlations exist between behavioral ratings of depressed patients and the fluctuations of their 17-OHCS levels; (4) through the intensive analysis of a few patients we have defined a number of characteristics of defense mechanisms which correlate

highly with changes in 17-OHCS levels; (5) we have shown that 17-OHCS levels accompany behavioral changes rather than precede or follow them; (6) we have used changes in 17-OHCS levels to study and locate specific days for analysis of precipitating events, and have characterized particular categories of precipitating events that seem relevant to depressive crises; (7) furthermore, we have used 17-OHCS levels to investigate the intense, intolerable psychic distress which is experienced by depressed patients and which is probably associated with the high suicidal rate in depressed patients. These findings and those described below have been outlined in more detail in previous reports and in a number of papers which are currently in press.

During the past year we have completed our analysis of two additional bodies of data. In general we have hypothesized that 17-OHCS levels reflect an increase in what we have come to call psychic distress or pain, and that this is particularly acute in depressive turmoil. Determinations of 17-OHCS appear to offer a tool for the study of stress, psychic pain, and defensive mechanisms. The first area of completed analysis during the past year is concerned with the study of a patient with 48-hour swings between mania and depression. This patient offers a rather striking case analysis as an illustration of the possible relationship between 17-OHCS levels and defense mechanisms.

Behavioral data in the form of descriptive paragraphs and two-hour ratings of mania by the nursing staff were collected on a continuous longitudinal basis throughout this patient's hospitalization. The patient's cycles persisted with clock-like regularity over a two year period of time and the manic days usually commenced abruptly at 3:30 a.m. 17-OHCS were analyzed and found to alternate regularly every other day in the opposite direction from 24-hour ratings of mania. On high, and at times wild, manic days, the 17-OHCS levels were low; and during the immobile, depressed days the 17-OHCS levels were high. Later during the course of her illness stable manic periods and stable depressed periods occurred; the manic periods were associated with stable low 17-OHCS levels and the depressed periods

with stable high 17-OHCS levels. The association of low 17-OHCS levels with mania appears to be a consistent finding supported by other investigations of individual cases. This patient's mania was characterized by intense denial of her illness and her problems, and it was felt that her denial comprised a critical characteristic of the manic behavior which was associated with low 17-OHCS levels. Depressed days associated with high 17-OHCS levels were characterized by feelings of suffering, pain and awareness of the severity and duration of her illness. Psychiatrists have suggested in the past that mania is a defense against the pain or distress of depression. Our findings are consistent with other investigators and may offer an interesting biochemical confirmation of this psychological theory. The work further suggests that the analysis of 17-OHCS levels may present a biochemical index which is sensitive to specific behavioral changes central to psychiatry, namely, changes in defensive organization.

During the last three years a significant proportion of our efforts have been concerned with the problem of the prediction of suicidal intent. Dublin estimates the incidence of suicide at about 25,000 deaths per year in this country. Suicide is the fourth most frequent cause of death in the productive ages of 18 to 45. It is relatively easy to identify some acutely suicidal patients. However, despite research and clinical skill, it remains difficult in many other cases for the general physician and even for the senior psychiatrist to predict which is the actively suicidal patient. Motto reviewed a series of 175 suicides and reported that 16% occurred within one week after seeing a physician. Extrapolating from Motto's figures and Dublin's estimate of the yearly suicide rate suggests that in this country approximately 4,600 patients see a physician each year within a week of the time they commit suicide. It is therefore clear that any possible aid to the general physician and even to the trained psychiatrist or analyst in the prediction of the potentially suicidal patient would be of great benefit. We have raised the question: "Could a biochemical test aid in the prediction of suicidal intent?" To our knowledge, this question has not

been previously reported in the literature. A recent paper reports serial urinary 17-OHCS levels, ratings of manifest suicidal behavior, and clinical data obtained during the hospitalization of three patients who subsequently committed suicide. Similar data are described for a comparison group of 33 depressed in-patients. The three patients who committed suicide showed high mean urinary 17-OHCS levels. The probability was .005 that these high levels could occur by chance in the group of depressed patients studied. All three suicidal patients showed relatively low ratings of suicide behavior and high mean 17-OHCS levels, thus suggesting the possible use of 17-OHCS levels in the detection of suicidal intent when it is not communicated by manifest behavior. Prediction of suicidal potential may be further aided by analyzing the 17-OHCS response patterns over time. Two out of three patients who committed suicide showed a pattern of sustained marked increase in 17-OHCS levels.

During the last three years a search has been made in this program for possible abnormalities in biochemical subsystems in depression in the hope that this would offer a clue to the etiology. One approach seemed to be to ask the question: "Do the three successful somatic treatments of depression, namely ECT, imipramine, and monoamineoxidase inhibitors have a common mechanism of action?"

Three areas currently offer promise. They are the areas of catecholamines, electrolyte changes and steroid metabolism. As described above, a good deal of effort has been applied to the study of steroids in depression. To date, the work has not extended into the field of electrolyte changes. During the past year Dr. John Davis and Dr. Bunney have completed a rather extensive review of the possible importance of norepinephrine in depression and have suggested a number of studies which they feel are critical in future investigations of this hypothesis. Briefly, they feel that in the last five years there has been a rapid growth in the information concerning the metabolism of the neurohumor norepinephrine. This information fits together the following observations: (1) Two classes of drugs—the imipramine (Tofranil) group and the monoamineoxidase inhibitor

group—are clinically effective in the treatment of many depressions. (2) Two drugs—reserpine and alpha-methyldopa—have been associated with severe depressive reactions in a significant number of hypertensive patients treated with these drugs. These observations may be related by a common mechanism of action. It has been discovered that all four drugs affect brain norepinephrine. The two groups of drugs useful in the treatment of depression functionally raise free brain norepinephrine while the two drugs which produce depression decrease brain norepinephrine. Many cases of depression produced by reserpine and alpha-methyldopa had histories of previous clinical depressions unrelated to drugs. This suggested that some patients who are prone to depression may be sensitive to depletion of norepinephrine. These data provide the basis for a testable hypothesis for present and future research efforts. It is hoped that an investigation of this theory may contribute to our understanding of the etiology of depression and may help put the treatment of particular sub-groups of depressed patients on a more rational basis.

Another area of study involves the intensive analysis of precipitating events which occur prior to the onset of depression. This should offer clues concerning the core problems of depression, plus giving basic information about response patterns of the depressed patients to stress. Forty patients and their relatives have been intensively studied to date. Factors have been distinguished related to external events, controlled and uncontrolled by the patient, and internal events. The temporal sequence of these events has been analyzed. The influential concept of the existence of a category of depression called "endogenous" is seriously challenged by these data.

Progress has also been made in the attempt to formulate theoretical concepts about the behavioral processes in the development of depressive illness, with an attempt to anchor these concepts in concrete observations of behavior. This work has been combined with the work on precipitating factors.

Two new areas of work have been initiated during the past year. The first has grown directly out of an interest in catecholamines in depression. This study will attempt to decrease

depressive symptoms in patients suffering from depressive reactions by treating them with L-dopa, a precursor of norepinephrine, and 5-HTP, a precursor of serotonin. This approach may help identify two subgroups of depressed patients, one which is sensitive to decreases of norepinephrine following alpha-methyldopa treatment and responds to treatment with L-dopa; and another group which responds to treatment with 5-HTP. The rating scales and other measures developed in the program provide a physiological, biochemical, and behavioral monitoring system to evaluate general, idiosyncratic, and part-function changes which may occur in the behavior of these patients following the treatment with these drugs.

Another study has been initiated in collaboration with Dr. David Horwitz of the Heart Institute, in which we are intensively interviewing a group of hypertensive patients who have become depressed on reserpine. It is interesting to note that again many of these patients have previous histories of clinical depressions unrelated to drug intake. Another study initiated this year is a collaborative study with Dr. Margaret Thaler Singer who is reviewing tapes of our depressed patients, on a blind basis, in an attempt to develop criteria for predicting those patients with suicidal intent. She is also attempting to further delineate those factors which are characteristic of patients with high steroid levels.

Finally, plans are being made to expand in two areas of this work. We hope to extend the studies of the prediction of suicidal intent through a collaborative study with the Los Angeles Suicide Prevention Center and thus be able to study a group of patients who are high suicide risks.

Also, we are currently developing techniques and theoretical concepts for further exploration of the relationship of steroids to psychopathology through the use of two drugs, dexamethazone and SU-4-885. With these drugs we will be able to directly study some of the effects of steroids on behavior and should be able to delineate some of the biochemical mechanisms involved in the maintenance of the extremely high steroid levels which have been observed in certain subgroups of depressed patients.

Section on Psychophysiology of Sleep

A constantly increasing mass of evidence now supports the thesis first elaborated by Dr. Frederick Snyder that dreaming is the subjective concomitant of a distinctive physiological state—a third basic biological mode of existence, of the same order, yet different from, sleep or waking. Although still pursuing some of the fundamental characteristics of this state, here referred to as the Rapid Eye Movement State (REMS), during the past year the Section on the Psychophysiology of Sleep under Dr. Snyder has given emphasis to several of its possible clinical implications.

The first extended longitudinal study of REMS in psychiatric patients has been completed and is now being prepared for publication. Studying all night polygraphic patterns of three psychiatric inpatients weekly over more than eight months, this study failed to support earlier anecdotal reports that the amount of REMS on any given night reflects subtle changes in psychological status. However, the single depressive patient in this group, showed some measure of positive correlation between degree of illness and both amount of REMS and rapidity of its onset after the beginning of sleep.

Depressive patients appear to be particularly suitable for such longitudinal studies, and the second phase of this work involves time sampling of sleep patterns for three day periods at various stages of this illness. For the first time this work provides objective evidence of the extent of sleep disturbance during the most severe stages of depression, and suggests that the degree of REMS deprivation entailed is relatively still greater than the marked curtailment of sleep itself. Although the possible relationship of REMS (dream) deprivation to psychopathology is now only theoretical, more intensive pursuit of these studies will search for associations between naturally occurring REMS deprivation during depressive crises and the onset of psychotic symptoms such as delusions and hallucinations.

Observations from many laboratories now indicate that the triggering mechanisms of epileptic seizures probably have differing relationships to brain function during REMS as com-

pared with sleeping or waking. In collaboration with the Electroencephalography Branch, NINDB, we have been taking advantage of a unique opportunity to study the activity of deeper structures of the human brain in relation to varying states of consciousness by recording the all-night sleep of neurosurgical patients who have had depth electrodes implanted in various brain structures for diagnostic reasons. To date these studies confirm the observations of others that seizure activity, which is abundant during sleep, is usually greatly reduced or absent during REMS. In some instances, however, focal seizure activity persists during REMS, and this may provide localizing information not otherwise available.

Earlier work here has shown that striking irregularity of vegetative functions, such as heart rate, respiration and blood pressure, is one of the distinctive characteristics of REMS. As a beginning approach to exploring the medical relevance of these findings, studies have been undertaken in collaboration with the National Heart Institute on the relationship of nocturnal angina to the regularly recurring periods of REMS. In the one suitable patient thus far studied over 13 nights, only one episode of angina out of 12 was unrelated in time of occurrence to the REMS periods. In addition to extending these studies to other important medical situations of episodic occurrence during sleep, plans are being made with the Heart Institute to apply an extensive battery of refined measures for the purpose of assessing circulatory dynamics during sleep and dreaming.

The great body of data accumulated during the past dozen years attests to the universality and relative constancy of the nightly pattern of alternating sleep and REMS, but there is still very little systematic evidence about naturally occurring factors which affect this pattern. It is of particular psychiatric relevance to determine whether stress or anxiety affects the occurrence of REMS. As an approach to this question we are exploiting an observation first made in the APB several years ago that for normal volunteers the experience of first arriving in a hospital is generally quite stressful and can be measured by elevations of urinary corticosteroids. With this in mind, subjects are

studied in the sleep laboratory for the first 3 nights of their stay here while 24-hour urine collections for corticosteroids are carried out, and the same procedure is repeated 6-8 weeks later, after they have become thoroughly acclimated to this environment. Since only two new subjects can be studied in this manner at each three-month turnover of normal volunteers, the collection of data proceeds slowly and assessment of it has not yet begun.

Still another type of study is aimed at further clarifying the normal characteristics of the REMS sleep pattern. Over the 6-8 hours that subjects ordinarily are studied in sleep laboratories, it is well established that initial REMS periods of the night are typically shortest, and that they tend to increase in duration as the night goes on. It is of interest, therefore, to determine whether this progressive lengthening would continue if subjects slept beyond the usual 6-8 hours, and many of our normal volunteers are obligingly capable of extending their sleeping period beyond this quota. Approximately 60 such nights have been recorded thus far, and a number of interesting findings are emerging. Surprisingly, the lengthening of REMS periods does not continue beyond the first 7 hours of sleep but levels off and even decreases. Another respect in which late morning sleep tends to resemble early night sleep is that high voltage delta patterns which traditionally are called "deep sleep" are more likely to be seen again in the 9th to 12th hours than during the 5th to 8th. In view of the well established observation that deprivation of REMS on one night is followed by increased levels on the next, it is of further interest that satiation of REMS by extended sleep does not appear to reduce the duration of it on the following night.

Our evolutionary studies of sleep and REMS have continued to utilize the opossum as the principal research subject. The most primitive of living animals in which the existence of REMS has been clearly demonstrated, this form spends a larger portion of its existence in this state than any other adult species yet studied, and indeed it prevails for as long or longer in each 24-hour period than does waking. Since REMS in the opossum might be expected to have prototypical characteristics,

current efforts are being directed to clarifying the factors which influence variations in its occurrence. By studying continuous 24-hour electroencephalographic recordings it has been possible to demonstrate diurnal changes, effects of sleep deprivation, and effects of adaptation to the experimental situation.

Although it may be that the physiological state associated with dreaming is a mammalian innovation, the possibility that the sleep of living reptiles might involve the same phenomena has not been fully explored. To that end, attempts have recently begun to extend the same type of approach to the study of the common iguana. It has yet to be determined whether reptiles manifest REMS, but by thus pursuing this state to its ultimate evolutionary source, we may hope to gain beginning understanding of its adaptive function in the organismic economy.

LABORATORY OF GENERAL AND COMPARATIVE BIOCHEMISTRY

During the last 12 months, the name of this Laboratory was changed from the Laboratory of Cellular Pharmacology to the Laboratory of General and Comparative Biochemistry. The present name reflects much more accurately the type of research programs being pursued in the laboratory, as well as the long-term interests of the senior staff. These research programs have continued, for the most part, along lines that had been developed in previous years and include four main areas of interest: (1) mechanism and pathways of protein biosynthesis, with special emphasis on the role of nucleic acids in the process; (2) biological methylation; (3) biological oxygenation; and (4) alkaloid biosynthesis.

One of the major efforts of the **Section on Proteins** has been the elucidation of the structure of soluble or transfer RNA (S-RNA). The work has concentrated on the structure of the specific RNA involved in transferring the amino acid, serine.

Although it has been clear for many years that the problem of determining the complete nucleotide sequence in any single species of S-RNA is an extremely complex one, developments in recent months indicate that the prob-

lem is even more complex than had been heretofore realized. Evidence has been accumulated which indicates that several different species of RNA can accept a single amino acid. Because of this development, the efforts of this section have been concentrated on attempts to separate different molecules of serine-specific S-RNA.

With this goal in mind, highly purified serine acceptor RNA from yeast has been obtained by partition chromatography of yeast S-RNA on Sephadex columns followed by chromatography on ion exchange (DEAE) columns. Results obtained with these methods provide evidence for two separate serine acceptor RNAs (as well as an inactive contaminant which is eliminated during the procedure). By use of these methods, processing of the large amounts of RNA needed for precise chemical work is facilitated and it is hoped that they will lead to characterization of the base sequence structure of serine S-RNA in the future.

A potential complicating factor in structural studies on S-RNA has been uncovered in separate experiments carried out with serine S-RNA from yeast. During countercurrent distribution separations of this RNA, two distinct peaks of serine acceptor activity have been detected. The most rapidly moving peak incorporates serine in the presence of only one enzyme, the serine-activating enzyme, while the slower moving peak, requires the presence of an additional enzyme, the S-RNA adenylate (cytidylate) pyrophosphorylase. These enzymatic studies and concomitant experiments on the incorporation of labelled ATP into the two serine S-RNAs clearly show the intact nature of the CCA terminal trinucleotide of the fast moving peak and the absence of the terminal adenosine moiety in the slower peak. The differential partition of the two S-RNA peaks may be due to the difference in terminal nucleotide structure although other differences in the molecules may be found. This discovery provides a method for separation of the two types of serine S-RNAs, a prerequisite for structural studies on a purified serine S-RNA.

Another important development in the Section on Proteins has been the purification of serine S-RNA synthetase in highly purified crystalline form. The serine S-RNA synthetase is currently being used to study the structural

requirements for S-RNA interaction with the enzyme. Such studies in a highly purified system may afford a useful model for the interaction of nucleic acids and proteins in general.

In the **Section on Cellular Regulatory Mechanisms**, work has continued on the mechanism of biological oxygenation reactions. As already reported, this work has culminated in the discovery of a new cofactor required for the enzymatic hydroxylation of phenylalanine. The work on the structure of this cofactor was completed last year when it was demonstrated that the active compound is a reduced biopterin derivative.

During the last 12 months, it has become increasingly apparent from work in this and other laboratories that reduced biopterin plays a fairly general role as a cofactor in a wide variety of hydroxylation reactions. Hydroxylating enzymes that have been resistant to study for decades have quickly become amenable to investigation now that it is apparent that reduced biopterin is an essential component of these hydroxylating systems. The list of biopterin-requiring enzymes now includes, beside the phenylalanine-hydroxylase, brain tryptophan hydroxylase, lipid cleaving enzyme, and tyrosine hydroxylase.

The latter enzyme has been studied extensively in this laboratory, to see if the cofactor participates in this hydroxylation reaction in the same manner in which it functions in the phenylalanine hydroxylating system. The results leave no doubt that the mechanism of action of the cofactor in the tyrosine hydroxylating system is identical to that already demonstrated for phenylalanine hydroxylase.

In another development in this section, it has been shown that the pure dopamine- β -hydroxylase is a copper protein. This represents the first demonstration of the essential role of this trace element in norepinephrine biosynthesis. Recent studies have also showed that the copper on the enzyme is intimately involved in the hydroxylation mechanism as an oxidation-reduction catalyst; it is reduced by ascorbate and is reoxidized during hydroxylation of the substrate.

These findings provide new insight into the intimate way in which vitamin C functions in

at least one enzymatic reaction. This system still represents the only metabolic reaction in which vitamin C is utilized; as of today, it is the only proven metabolic role for ascorbate at the enzyme level.

The work in the **Section on Alkaloid Biosynthesis** has resulted this year in several significant advances. If one area must be stressed, some of the results from the investigations on homocystinuria may be selected. The findings of a year ago that suggested the basic lesion in this disease is a defect in cystathionine synthase have been confirmed in other patients. Sufficient control studies have been carried out to enable us to state that both parents of the proband have only 35-40 percent of the mean control activity of cystathionine synthase so that the disease is transmitted as an autosomal recessive trait. The improved assay now available promises to enable us to measure accurately the residual enzymatic activity present in homocystinuric patients. It is hoped that measurements of this activity can be correlated with a description of the clinical status of the patient. In particular, attempts will be made to determine whether a residual activity of 10-15 percent is critical to the prevention of mental retardation, a question raised by the finding that a homocystinuric patient who is not mentally retarded has this level of enzyme activity. The evidence that cystathionine synthase activity is missing in the brain of a homocystinuric patient is the first instance to our knowledge in which a metabolic disease which leads to mental retardation has been demonstrated to be associated with a specific enzyme defect in the brain itself. The study of threonine dehydratase activity in cystathionine synthase deficient patients is promising since it may ultimately throw light on those factors which govern enzymatic specificity. Investigation of patients with cystathionine synthase deficiency has allowed us to draw the following conclusions as to the normal human: (a) Cystathionine is an obligatory intermediate in the conversion of the sulfur of methionine to sulfate; (b) cystathione synthase activity is unaffected by a drastic change in the level of its substrate. Both of these conclusions are helpful in understanding normal mammalian metabolism of the sulfur amino acids. It is hoped that the continuing study of

the products formed from methionine will also contribute new knowledge to this area of metabolism.

LABORATORY OF NEUROCHEMISTRY

During fiscal 1965 the laboratory made a number of significant scientific discoveries, the essential nature of which was the proposal of molecular-level models which account for puzzling features of corresponding life processes:

A quantitative theory of the recognition process in the translation of the genetic code has been proposed (M-NC-10 and 37), the theory explains observed degeneracies and ambiguities, predicts new ones and suggests a general system for controlling the reading of the genetic code. A model for the binding of messenger RNA to ribosomal RNA has been proposed on the basis of experiments using dye-binding methods developed in this laboratory. A monopole method (M-NC-10, 1964) for calculating forces between molecules was shown to be of considerable importance in solving biological problems.

Ordered helical structures were proposed for certain important biological polysaccharides on the basis of their optical activity dispersion curves (M-NC-21). Helicity has proven to be very important in determining the biological functions of proteins and nucleic acids. We anticipate that this will also be true for this third class of biopolymers.

The precise location of small molecules which are attached to the gene-substance DNA was proposed on the basis of X-ray diffraction and other physical techniques (M-NC-22). It is common knowledge that alterations in DNA structure by chemical agents can result in mutation, cancer, growth, and differentiation. Studies such as these are necessary to determine how one agent which binds to DNA can affect growth, e.g., the rate of cellular protein synthesis while another can cause mutations, e.g., affect the kind of protein being synthesized.

A general theory of the optical properties of biopolymers has been proposed (M-NC-27). This is a very important development since such optical properties are being used daily on

a strictly empirical basis by hundreds of biochemists to provide information about structure and reactivity of biopolymers. Because these properties of biopolymers are recognized to be highly relevant to the role these polymers play in many biological processes, a sense of urgency has been generated which compels scientists to utilize information provided by optical studies, despite the strictly empirical nature of the relations established between them. The present theoretical treatment is necessary to establish these relations on firmer footing.

It was proposed this year that of the two genes known to be related to γ G-immunoglobulin synthesis, the effects of the b and a loci are on the amino acid sequence of the light chain and heavy chain respectively (M-NC-31). The most important unsolved puzzle in the field of immunology is the mechanism by which antibodies specific to thousands of different compounds can be formed from relatively simple proteins. The present work shows the manner in which the principal degree of freedom of the proteins, their amino acid sequences, is under genetic control. As is the case of other projects in this laboratory our long range goal is to delineate the physical forces whose operation accounts for the recognition by one molecule (e.g., antibody) of another (e.g., antigen) and the interaction appropriate to this recognition (e.g., precipitation if correct antibody-antigen combination; no precipitation if incorrect combination).

A computer program for reconstructing sequences of proteins and nucleic acids has been reported (M-NC-35). With this program a computer can perform in a matter of several minutes the millions of logical steps required to build a sequence out of its fragments without making a single inconsistent or ambiguous logical decision. The importance of this development lies in the fact that although several years ago knowledge of polymer sequences was only of intrinsic interest, today knowledge of specific sequences is of critical importance in testing theories of the genetic code and enzyme action. It has therefore become important that the program developed in this laboratory be used to prove that each proposed sequence is actually consistent with the experimental data

and, more important, is the only sequence consistent with the data.

A system for predicting the hallucinogenic activity of as yet untested compounds has been proposed (M-NC-35). This is of importance in offering a rational sequence in which to synthesize new drugs: Out of the countless compounds which might be synthesized at random, the theory selects a small set as the most promising and which therefore should be tried first. The synthesis and experimental testing of the predicted compounds is also of importance as a test of the fundamental quantum mechanical theory on which the system is based. If the theory proves to be correct we may have found a very close connection between consciousness and molecular activity.

Since this molecular approach has proved to be so productive in areas of biology where it has been applied, we have devoted a considerable amount of effort during the year to an analysis of whether it might fruitfully be applied in the future to neurochemistry: we invited scientists from outside the laboratory to discuss this subject. Each staff member reviewed his own research plans for the next decade to see how they relate to mental health research. Several teams of scientists were established to examine in depth the most promising areas for future research. A ten-year plan for the laboratory was drafted.

It was generally agreed that neurochemistry can play an important role in developing our understanding of brain and behavior if it evolves from emphasis on chemical analysis and stoichiometry toward dynamic molecular models. Such models are elegantly suited for both the quantitative interpretation of past experiments and prediction and control of future results.

There must be a certain minimum body of anatomical and biochemical information available about a system before molecular-level models can be meaningfully constructed. Within the next decade such information will be available for many important processes of the nervous system. In fact, a number of senior investigators in the Institute have already refined their biological experiments to the point where model building at the molecular lev-

el becomes of critical importance in designing further experiments.

There are areas outside of neurochemistry and closer to the center of current biological research where sufficient detailed biochemical information is available at present to permit the construction of molecular-level models. We feel that it is most productive to concentrate our efforts on those problem areas which seem ripe for treatment by molecular-level analysis, in which areas we have sufficient background and competence, and where, if the area has been worked over unsuccessfully before, we have new ideas, regardless of whether the area can properly be called neurochemistry. Naturally, we intend to watch for new developments in brain and behavior which satisfy these criteria and to work on them if such should occur. However, we anticipate that, in general, for this field to reach full fruition, the center of gravity of biological science must move away from its present concern with the genetic code, into and beyond the fields of development, aging, and cancer, and finally toward brain and behavior. At each stage in the evolution of the science of biology there will appear problems solvable only by the use of molecular-level analyses and concepts. As future problems evolve into current problems we hope to contribute to each one, always keeping in mind the goal of ultimately contributing to brain and behavior. The thread of continuity running through the actual programs carried out in Fiscal 1965 is this preoccupation with the understanding of puzzling biological problems through the development of dynamic, molecular-level models. We have applied this kind of analysis to problems as diverse as the genetic code, hallucinogenesis, immunology and histology.

LABORATORY OF CLINICAL SCIENCE

The Laboratory of Clinical Science was organized in 1955, bringing together competences in clinical psychiatry and in the basic disciplines of physiology, pharmacology and biochemistry. Dr. Edward Evarts, who was the first chief of the Laboratory, left the post in 1956 in order to pursue more intensive research in neurophysiology and has remained as chief of that section. He was succeeded in 1956 by

the present chief of the Laboratory. Since the current year represents the tenth anniversary of the establishment of the Laboratory, it may not be inappropriate to review the work of the Laboratory over that period of time.

The Laboratory has not seen its role as providing "a concerted attack on a large and complex problem"^{*} such as mental illness. Instead, great reliance has been placed upon the senior scientists to develop their research programs individually and to the fostering of originality and high standards of scientific excellence.^{**} Where it has seemed appropriate to do so, the interaction of biological and somatic variables with the psychological and social features of the clinical picture have been investigated intensively. More often it has seemed the wiser course to explore and elucidate the biological processes themselves in the conviction that strong bridges require firm foundations.

Some of the work of the laboratory has had direct relevance to problems in clinical psychiatry. The studies of Sokoloff and Klee have provided an explanation for the retarded mental development found in cretinism. Studies conducted here in a broad spectrum of elderly individuals have provided a basis for a primary role of cerebral circulatory insufficiency in many of the psychoses of the senium. In the field of the schizophrenic illnesses, the laboratory has played a leading role in demonstrating the importance of non-disease variables, including institutionalization, vitamin deficiencies and the results of previous infections, in research on biological aspects. Its studies with certain amino acids and amines have contributed significantly to current hypotheses of the etiology and pathogenesis of schizophrenic ill-

* Biomedical Science and Its Administration: A Study of the National Institutes of Health. Implementation of plans and policies through the direct operations and the intramural programs, page 36.

** Ibid. Biochemistry Panel Report, page 145: "Crash programs in research have been almost uniformly unsatisfactory and very expensive. One of the most important ingredients for successful research is the choice of the problem that is ready for attack. Often a critical advance arising from basic research opens the door. Premature efforts lead to disappointments and frustrations and retard rather than accelerate progress."

ness. A study on schizophrenia in adopted children being conducted in Denmark in collaboration with the Laboratory of Psychology may yield new information on the nature-nurture interrelationships in that group of disorders. Research on thyroid function in episodic psychoses by Durell and his collaborators has revealed significant disturbance in thyroid and pituitary function in the non-psychotic prodromal state. The work of Axelrod and of Kopin and their collaborators has made possible definitive studies of epinephrine and norepinephrine secretion and metabolism in psychiatric illness and in the action of psychotropic drugs. The studies of Glowinski, Axelrod and Kopin offer an explanation for the action of antidepressant drugs on the brain. The demonstration by Dr. Kies of the protein antigen in experimental allergic encephalomyelitis has important implications in the etiology and pathogenesis of demyelinative diseases such as multiple sclerosis.

Scientists in the laboratory have been responsible for a number of noteworthy advances in fundamental knowledge pertaining to the nervous system. Dr. Evarts, who has pioneered in studies of the activity of single neurons, has demonstrated and characterized the differential aspects of cortical neuronal activities in various stages of sleep and wakefulness. Dr. Sokoloff and his collaborators have demonstrated the important action of thyroid hormone on protein synthesis which appears to be the mechanism for its physiological actions, and have elucidated the specific steps in protein synthesis at which the action occurs. They have demonstrated a specific effect of thyroxine on protein synthesis in the immature brain which promises to support and explain the role of that hormone on dendritic proliferation in the normally maturing cerebral cortex. Dr. Axelrod has delineated the pathways by which the body metabolizes the important hormones, epinephrine and norepinephrine, described the crucial enzyme in the inactivation of these hormones, and demonstrated the action of a large number of neurotropic drugs on the storage, release and inactivation of these hormones. His work may truly be said to have revolutionized the field of catecholamine research. Kopin and his collaborators have contributed significantly to the mechanisms of storage of noradrenaline

at the sympathetic nerve endings and its release by sympathetic activity and by the action of a number of drugs. By demonstrating the accumulation of octopamine there under the effect of monoamine oxidase inhibitors and its release by sympathetic nerve activity, they have probably explained the paradoxical but important hypotensive effects of these agents. Glowinski, Axelrod and Kopin have succeeded in studying the metabolism of norepinephrine in the brain and demonstrated important actions of the psychotropic drugs on the release of brain norepinephrine which promises to contribute significantly to an understanding of the role of that substance in cerebral function and behavior. Wurtman and Axelrod have adduced compelling evidence for an endocrine function of the pineal gland and they and their collaborators have shown an important role for that structure in a number of biological rhythms. When the account of the biological processes of importance in mental illness and its treatment is finally completed, it is our hope that some of the more significant chapters will have been written by scientists in this Laboratory.

In addition to its primary responsibility in psychiatric research, the Laboratory takes very seriously its role in the postdoctoral education and training of young investigators for careers in psychiatric research and in cognate disciplines. More than 100 such fellows from the United States and abroad have received substantial training in the Laboratory. The senior investigators in the Laboratory, some of whom hold academic appointments, lead seminars at the NIH and give a large number of lectures at universities throughout the country.

The Laboratory is represented by membership in the National Academy of Sciences and in the national societies representing each of its disciplines, and by membership on the editorial boards of numerous journals. Members of the Laboratory played an important role in the establishment of the Psychiatric Research Society and the Journal of Psychiatric Research, which have exerted salutary influences on the field.

The progress of the work in the various sections during the course of the current year is examined more specifically and in greater detail under the various headings which follow.

Section on Cerebral Metabolism

The research activities of the Section on Cerebral Metabolism under Dr. Louis Sokoloff during the past year have continued in the areas of mechanism of action of thyroid hormones, the relation of this mechanism of action to the different responses of mature and immature brain to thyroxine, and the role of thyroid hormones in the biochemical processes underlying development and maturation of the brain.

Previous work of this Section has demonstrated that thyroxine pretreatment *in vivo* or its addition *in vitro* stimulates the rate of amino acid incorporation into protein in cell-free rat liver preparations. Thyroidectomy results in a reduction in this rate. The thyroxine stimulation has been localized to the step in protein biosynthesis involving the transfer of sRNA-bound amino acid to protein. The thyroxine effect is characterized by an absolute requirement for mitochondria and an oxidizable substrate in the reaction mixture, and a lag period of approximately five minutes precedes the stimulation. It has been clearly demonstrated that it is not thyroxine itself but some product of an interaction between thyroxine and the oxidizing mitochondria formed during the lag period which is responsible for the stimulation. ATP, GTP, and reduced glutathione, the only cofactors known to influence the step in protein synthesis stimulated by thyroxine, have all been excluded as the possible active intermediate. An active intermediate has in fact been prepared in soluble form, and during the past year considerable efforts have been directed toward its isolation, purification, and identification. Its identity is still unknown, but a great deal has been learned about its properties and the requirements for its formation. These studies will be continued in the coming year.

The mechanism of the stimulation of protein biosynthesis by the active intermediate has also been under investigation. Previous work in this Section demonstrated that changes in microsomal membrane permeability were not involved since similar effects were observed with isolated ribonucleoprotein particles in place of microsomes. During the past year studies were concluded clearly proving that the thyroxine stimulation of protein biosynthesis is primary

to the reported effects of thyroid hormones *in vivo* in thyroidectomized animals on messenger RNA synthesis. Administration of a single dose of thyroid hormones *in vivo* was found to stimulate protein biosynthesis within two hours, the shortest time examined. This is by far the earliest effect of thyroid hormones *in vivo* thus far reported and precedes the alleged effect on RNA synthesis by four to six hours. Furthermore, thyroxine *in vitro* has been found to stimulate amino acid incorporation into protein in the absence of any messenger-RNA synthesis and in fact to stimulate synthetic messenger-RNA (i.e. poly U) directed amino acid incorporation into artificial poly-peptides, even when the synthetic polyribonucleotide is added in excess. These results indicate that the primary effect of thyroxine on protein biosynthesis is to stimulate the completed protein biosynthetic machinery to work more rapidly or effectively and not to stimulate the formation or completion of more protein biosynthetic machinery. It is, of course, possible and reasonable that in intact, organized, and integrated cellular systems with regulatory feedback mechanisms in operation, the second effect may result as a secondary consequence of the first effect.

The important question whether the thyroxine effect on protein biosynthesis is general or confined only to very special proteins has been partially resolved by the finding that thyroxine added *in vitro* to a combined rat liver mitochondrial and rabbit reticulocyte cell sap and ribosomal system stimulates the rate of amino acid incorporation into both the α and β chains of hemoglobin being synthesized by the reticulocyte ribosomes. This finding represents the first demonstration of a thyroxine effect *in vitro* on the synthesis of a known protein. Since the thyroxine effect has now been observed in infant brain, liver, and reticulocyte preparations, all of which synthesize different types of proteins, it would appear that the thyroxine effect is fairly general. Furthermore, since it is known that the amino-terminal amino acid (the first one to be laid down in the synthesis of a protein molecule) of both the α and β chains is L-valine, it is now possible to determine whether thyroxine stimulates the initia-

tion of the chain synthesis, the completion of the polypeptide chain, or both.

It was previously observed that amino acid incorporation into protein in immature brain preparations is more rapid than in mature brain preparations. Also thyroxine stimulates amino acid incorporation in the immature brain but not in mature brain. Both differences are the results of some functional dissimilarities between mature and immature brain mitochondria. Another functional difference between mature and immature brain mitochondria has now been observed. Immature brain mitochondria contain the enzyme, β -hydroxybutyrate dehydrogenase, which remains active during the period of maturation of the brain and then rapidly declines to negligible levels. The exact function of this enzyme is not known, but it is believed to be involved in lipid synthetic or metabolic pathways. Investigations are under way to determine if this enzyme plays any part in the biochemical process related to maturation of the brain.

In the course of studying mitochondrial protein biosynthesis in brain, it was observed that impure preparations of mitochondria from immature brain contaminated with myelin and nerve endings also incorporate amino acids *in vitro* into peptide linkage in the proteolipid of myelin. This finding offers the possibility of studying the mechanisms of myelin synthesis *in vitro*, and studies along these lines are being carried on.

The studies currently in progress and being planned for next year are along the lines indicated by the open questions and gaps in our knowledge pointed out in the discussion above.

Section on Physiology

Within the past year the Section on Physiology under Dr. Edward Evarts has been concerned with two major problems. The first of these has involved an analysis of the relation between the size of a neuron and the change in the discharge frequency of this neuron with sleep. The second problem has been undertaken just recently and involves an analysis of the role of the cerebral cortex in the initiation and control of voluntary movement. These two

areas of investigation will be discussed in the paragraphs to follow.

Sleep

The previous work of this Section had made it clear that sleep is not a state in which there is a generalized reduction of discharge of cerebral neurons. Recordings of single unit activity in a number of regions of the brain had shown that natural sleep (in contrast to anesthesia) is associated with increased discharge frequency in certain neurons and decreased discharge frequency in others. Given this fact, the question arose as to what functional or structural properties differentiate neurons which are more active during sleep from neurons which are less active. An attempt to provide an answer to this question for pyramidal tract (PT) neurons was made in this study. For pyramidal tract neurons it is possible to estimate axonal conduction velocity from the latency of the antidromic response to stimulation of the medullary pyramid. Conduction velocity is closely related to axonal diameter and axonal diameter is closely related to cell size. As a result of these relations, it is possible to infer relative neuronal size from relative antidromic response latency: the smallest neurons have the shortest antidromic response latencies.

In recordings from the motor cortex of the monkey it was found that the largest PT neurons are relatively inactive during waking without movement (W) and that they speed up from W to sleep (S). In contrast, the smallest PT neurons are almost continually active during W and slow down from W to S. This relation between cell size and change of discharge frequency from W to S may be viewed both teleologically (small cells may run down metabolically and *need* inactivity) and in relation to the differing functional roles of large and small cells. Thus, the large cells which are inactive during waking *without* movement are extremely active when the monkey *moves*. The small PT cells, in contrast, tend to be tonically active throughout W and to transmit information concerning steady-state aspects of the central nervous system's motor output. In view of this functional correlate of size in PT neurons, it is not yet clear whether the differential

effects of sleep in relation to cell size are primarily or secondarily a function of the structural difference. When techniques become available for determining the relation of sleep and waking to the metabolic and ionic status of individual neurons, it may be possible to determine precisely *why* smaller neurons tend to slow down with sleep and larger neurons tend to speed up.

Movement

Studies of the role of the cerebral cortex in movement have, in large part, been based upon the use of two techniques: the first of these techniques, that of cortical ablation, has allowed delimitation of the major cortical motor area in a number of animals. In man, observations of the effects of cerebral tumors and lesions have allowed neurologists to locate the motor cortex. A second technique which has been successfully employed in delimiting the extent of the motor cortex has involved observations of the effects of electrical stimulation of the exposed cerebral cortex in man and lower animals. Though these two techniques (ablation and stimulation) have been useful in outlining the general areas of cortex which are especially important in movement, the crudeness and unphysiological nature of both techniques severely limits the extent to which they can provide knowledge of the role of cortical neurons in *normal* movement. The ablation technique can tell us the location of areas whose removal produces paralysis but it does not indicate how the ablated cortex normally functions. Electrical stimulation, on the other hand, causes millions of cortical neurons to discharge simultaneously and in a spatio-temporal pattern which never occurs normally. Only during an epileptic fit would such massive discharges occur.

The present work of the Section on Physiology involves the application of a third technique to the problem of finding out how the cerebral cortex initiates and controls movement. We hope to determine the way in which information is coded and transmitted from a variety of cortical regions to lower centers in such a way as to produce motor behavior. At the beginning of these studies on movement atten-

tion is being centered on the pyramidal tract, because it is the main output path from the cortex to the spinal cord. Pyramidal tract neurons in the precentral gyrus of the monkey were identified by their antidromic responses to electrical stimulation of the medullary pyramid. Units with short antidromic response latencies (and therefore large axons) tended to be phasically active during contralateral arm movement and to be relatively inactive in the absence of movement. Units with long antidromic response latencies (and therefore small axons) tended to have tonic, regular discharge in the absence of movement and showed both upward and downward shifts in discharge frequency with movement. Increase in over-all amount of discharge with movement was greatest for the shortest latency units and least for the longest latency units. With sleep, the clear differences between discharge patterns of long- and short-latency units disappeared, both types of neurons discharging in sporadic bursts.

This relation between structure and function in PT neurons is similar to the relation between the structural and functional properties of α -motoneurons in the mammalian spinal cord. Eccles et al. have found that the average conduction velocity of soleus motoneuron axons is 72% of that for gastrocnemius motoneurons. The α -motoneuron fibers to gastrocnemius (a pale, phasically active muscle) have correspondingly larger diameter than the fibers to soleus (a red, tonically active muscle). Granit and co-workers have emphasized the relationship between structural and functional properties in α -motoneurons, and have presented several lines of evidence that tonically active α -motoneurons are smaller than phasically active α -motoneurons. The present findings, then, indicate that a basic functional property of the spinal cord motoneuronal system has been duplicated in the pyramidal system. This relation between axonal size and function occurs rather frequently. To cite another example, the large Ia afferents of the nuclear bag fibers in the muscle spindle carry information which is particularly related to transients, whereas the smaller group II afferents of the nuclear chain fibers tend to carry steady-state information. The efferent neurons to the intrafusal fibers likewise show this relation between function

and structure, the larger gamma-1 fibers innervating the phasically active nuclear bag fibers, and the smaller gamma-2 fibers innervating the more slowly contracting nuclear chain fibers. Often, then, large fibers with high conduction velocity carry transient information and are phasically active, whereas small fibers with low conduction velocity carry steady-state information and are tonically active.

Section on Pharmacology

Since the Section on Pharmacology was established under Dr. Julius Axelrod in January 1955, it has been concerned with three areas of research: (1) the physiological disposition and metabolism of drugs and hormones; (2) enzymes involved in the formation and metabolism of drugs and physiologically active compounds; and (3) the biochemical mechanisms of action of drugs. This Section has studied physiological disposition and metabolism of narcotics, hallucinogens, biogenic amines (noradrenaline, adrenaline, serotonin, histamine) and melatonin. About 20 new metabolites and 10 normally occurring compounds have been found. A few of these compounds have proved useful in the diagnosis of pheochromocytoma, neuroblastoma and pinealoma. A biochemical lesion in nonhemolytic nonobstructive jaundice and new ideas concerning the fate of the neurotransmitter in the sympathetic nervous system have also been described. Fifteen new enzymes have been discovered in this laboratory, several of which have been shown to be important in the metabolism of glucose, drugs (morphine, codeine, papaverine, LSD) and in the metabolism and formation of hormones (corticoids, noradrenaline, melatonin) and physiologically active compounds (histamine, serotonin). The enzymatic formation of psychotomimetic compounds from normally occurring substances has also been demonstrated. A mechanism for the development of tolerance to narcotic drugs has been proposed which stimulated much controversy and further work in this area. Biochemical mechanisms for the development of tachyphylaxis and denervation supersensitivity have been described. Also, the action of cocaine, sympathomimetic amines, cardiovascular and antidepressant drugs on the

uptake, release and metabolism of catecholamines have been demonstrated. In the past two years the laboratory has been mainly occupied with studies on the pineal gland. It has been shown that melatonin, a compound made in the pineal gland, is a hormone that acts on the gonads. The formation of this hormone was shown to be regulated by environmental lighting. The pathway by which lighting reaches the pineal gland is via the sympathetic nervous system. A considerable part of the productivity of the Section on Pharmacology is due to the enthusiasm and imagination of a substantial number of research associates and visiting scientists who have spent a year or more in the laboratory.

In the past year it was demonstrated that the pineal gland can serve as a biological clock. There is a circadian rise and fall of hydroxyindole-O-methyl transferase. This enzyme makes melatonin and is present only in the pineal gland of mammals. This rhythm is completely controlled by environmental lighting and the information about this lighting is transmitted by means of the sympathetic nerves. The circadian rhythm in serotonin concentration in the pineal gland has been found to be endogenous. The pineal gland receives information about this rhythm from the central nervous system via sympathetic nerves. The activity of 5-hydroxytryptophan decarboxylase in the pineal gland was found to be influenced in environmental lighting. Changes in the activity of this enzyme induced by lighting are controlled by the sympathetic nerves innervating the pineal gland. Melatonin has been previously shown by others to be the most potent pigment lightening agent known in amphibians. We have demonstrated the enzymatic formation of this hormone in the amphibian brain. Hydroxyindole-O-methyl transferase, melatonin and serotonin were detected in a metastatic parenchymal pinealoma. These findings make possible the detection of these tumors.

Because of the small size of the pineal gland it was necessary to devise sensitive procedures for the measurement of enzymes and amines. Specific and highly sensitive methods have been developed for the assay of monoamine oxidase, 5-hydroxytryptophan decarboxylase, hy-

droxyindole-O-methyl transferase and serotonin. Because of the high proportion of sympathetic nerves in the pineal gland, investigators in this laboratory were able to supply direct evidence for the presence of monoamine oxidase in these nerves. Work on the pineal gland was carried out in collaboration with Drs. Snyder and Wurtman.

The laboratory has been involved in studies on the metabolism of catecholamines in the brain and the effect of centrally acting drugs. The investigations were made possible by the elegant techniques of introducing radioactive catecholamines into the brain which were devised by Dr. Glowinski, a visiting scientist in this laboratory. H^3 -noradrenaline can be taken up and stored in more than one compartment in the brain. It is then slowly released and metabolized. Reserpine appears to release H^3 -noradrenaline intraneurally so that it is metabolized by monoamine oxidase within the sympathetic nerve. Amphetamine releases noradrenaline rapidly from the brain neurons so that it is metabolized mainly by catechol-O-methyltransferase. Anti-depressant drugs such as imipramine and amphetamine block the uptake of noradrenaline into the brain neuron and thus prevent the inactivation of this amine.

After the administration of H^3 -noradrenaline there was negligible uptake of the radioactive hormone in the hearts of immunosympathectomized rats. There was a partial reduction in the uptake of H^3 -noradrenaline in the salivary gland and normal uptake in the gut and gonads. In view of previous work in this laboratory, these results suggest that the sympathetic nerves in the immunosympathectomized rat heart were not fully developed while they are partially or fully formed in the salivary gland, gut and gonads.

A relationship between the ability to inhibit histamine N-methyl transferase and electronic configuration of the inhibitory compound as determined by molecular orbital calculation has been found. These results provide a new approach to the study of enzyme mechanisms. Sex differences in the enzymatic N-methylation of histamine and its control by sex hormones have been observed. A more rapid rate in the enzymatic N-methylation of histamine was found in male rat kidney than in female kid-

ney. The reverse has been found in mice kidneys. This sex difference in enzyme activity appears to be influenced by male gonadal secretions. The sex differences in the urinary excretion in histamine may be explained in histamine N-methyl transferase activity.

LSD has been shown to be unequally distributed in the brain. It is highly localized in the subcortical visual areas, the limbic system and the hypothalamus. Molecular orbital calculations have been performed on a number of hallucinogenic drugs. A close correlation between hallucinogenic potency and the energy of the highest occupied molecular orbital has been found. Using this relationship it is possible to predict the structure of hallucinogenic compounds.

H^3 -Estradiol has been found to be selectively taken up in target organs and in certain areas of the brain. There is a saturable mechanism for the uptake of H^3 -estradiol in these tissues. Anti-fertility drugs block this uptake and binding of H^3 -estradiol in target tissues and in the hypothalamus.

Section on Medicine

The investigations during the past year of the Section on Medicine under the leadership of Dr. Irwin Kopin have concerned the synthesis, storage, release, metabolism, and mode of action of the biogenic amines and modification of these by nerve impulses, aging, drug treatment and endocrine and electrolyte status.

Previous work in this laboratory has dealt with the quantification of the routes of metabolism of catecholamines, the relationship of these metabolic pathways to the subcellular localization in various pools of the adrenergic transmitter and to the mode of release of norepinephrine from its binding sites. It was shown that intravenously administered catecholamines or those released into the blood stream by sympathetic nerve activity or administration of sympathomimetic drugs such as tyramine are mostly O-methylated. Catecholamines which are bound and released intraneuronally, either spontaneously or consequent to the action of drugs which diminish the binding capacity in particulate storage sites (such as reserpine), are mostly deaminated without ever

becoming active. MAO inhibition prevents this destruction, results in liberation of free norepinephrine outside the neuron and consequently reverses the action of reserpine. Various endocrine, pharmacological, and pathological states have been studied in relation to their effects on synthesis, storage, and metabolism of the catecholamines.

The rate of norepinephrine synthesis is examined by studying its formation from various labeled precursors of the catecholamine, or by studying the rate of decrease of its specific activity after labeling the store with exogenous norepinephrine. Disulfiram inhibits dopamine β -oxidase and has been shown to diminish norepinephrine synthesis. Tyramine releases norepinephrine from its storage sites and with repeated doses of this drug norepinephrine synthesis increases, and "escape" from tyramine tachyphylaxis may occur. This can be prevented by treatment with disulfiram. These observations are the first evidence that norepinephrine synthesis may be increased as a result of catecholamine depletion.

A number of phenylethylamine derivatives have been found to be taken up by sympathetic nerve endings and converted to their β -hydroxyl derivatives which are retained in the same subcellular fractions as endogenous norepinephrine. These observations have led to two large areas of investigation—the chemical nature of norepinephrine binding and a study of false adrenergic transmitters.

The demonstrated ability of phenylethanamine derivatives to enter and be retained at norepinephrine storage sites suggested that these substances could be used as models to determine the relative importance of the chemical groupings in the norepinephrine molecule for its binding. It was found that particulate retention could be rank ordered: norepinephrine > dopamine > *m*-octopamine > *p*-octopamine, while amines without catechols or β -hydroxyl groups were not retained at all. This *in vitro* rating correlated with the ability of drugs such as tyramine, reserpine, and guanethidine to release these amines *in vivo*. The stronger the affinity for particulate binding, the greater the resistance to depletion. Both the β -hydroxyl

group and the catechol appear involved in binding of these amines.

Those amines which are retained in the particulate fraction were found to be released by sympathetic nerve stimulation. This group of "false neurochemical transmitters" provides evidence that amines are released from vesicles rather than the soluble cytoplasmic fraction. The demonstration that certain of these substances, *p*-octopamine and *m*-octopamine, can replace norepinephrine led to a hypothesis explaining some seemingly paradoxical effects of monoamine oxidase inhibitors. These drugs interfere with destruction of amines and result in increased tissue levels of norepinephrine. Clinically, however, they produce an apparent adrenergic blockade. The demonstration of intraneuronal accumulation of octopamine in animals treated with MAO inhibitors provided a basis for an explanation of the clinical effects of these drugs. Octopamine in the sympathetic nerve vesicles replaces a portion of the norepinephrine, and, in spite of elevated norepinephrine levels in the tissues, replaces a portion of the catecholamine released during sympathetic nerve stimulation. Since octopamine is almost completely inactive, the nerve impulse is less effective, although tissue levels of norepinephrine may be elevated.

It is not true, however, that all substances which accumulate in sympathetic nerves and are released by nerve stimulation replace norepinephrine. Bretylium-H³ accumulates in the sympathetic nerves and can be released by nerve stimulation. Unlike norepinephrine, however, it is not released by acetylcholine and it is not bound in the sympathetic nerve vesicles. Bretylium prevents release of norepinephrine by the sympathetic nerve impulse, but not by acetylcholine.

These observations suggest that bretylium interferes with sympathetic nerve function by replacing a substance released as an intermediate between nerve impulses and norepinephrine release. This may be the first direct evidence for the release of intermediate compounds during the conversion of the sympathetic nerve impulse into norepinephrine release.

Studies in man are being performed to estimate the rates of production and pathways of metabolism of the catecholamines in normal in-

dividuals and in patients with disorders of the central and autonomic nervous system. Methods are now available in our laboratory for estimation of several of the metabolites of the catecholamines and these are being used in combination with radioactively labeled catecholamine infusions for estimation of turnover rates, binding and release rates, etc.

Investigations in patients with familial dysautonomia have led to observations of abnormalities in several sensory systems—taste, hearing, perception of hot, cold and pain, and abnormalities in cardiovascular as well as peripheral reflexes. These abnormalities are being investigated in collaboration with some physicians in the National Heart Institute. Both patients and their mothers appear to excrete excessive amounts of homovanillic acid, a metabolite of dopamine. The patients excrete lower amounts of VMA, the major norepinephrine metabolite. Investigations are being planned to determine the cause of these abnormalities and their relationship to the clinical syndrome.

Methylation is a major pathway of metabolism of the catecholamines and other amines. The addition of a methyl group alters the activity of the compound and may result in activity increase, decrease, or may impart entirely new properties to the molecule. S-adenosylmethionine is the major methyl donor in mammals and a method of assay of this key intermediate has been developed by Drs. Baldessarini and Kopin. Methionine increases SAMe levels while methyl acceptors diminish the concentration of this intermediate. Levels are higher in infant liver and brain than in adults. The levels are elevated in leukemic white blood cells and depressed in livers of animals with a portacaval shunt, presumably because dietary methionine fails to reach the liver. The dynamics of activation of methionine are currently being studied.

The control of histamine synthesis in various states known to be associated with gastric hyperacidity and peptic ulcer has also been the subject of investigation. Alterations in the levels of the enzyme responsible for histamine synthesis, histidine decarboxylase, appear to correlate with the increased histamine levels and hyperacidity produced experimentally in rats by portacaval shunt and corticoid adminis-

tration. The effects of drugs which inhibit histidine decarboxylase are being studied.

Section on Biochemistry

A major program of the Section on Biochemistry under Dr. Marian Kies has centered on experimental allergic encephalomyelitis, and experimental animal model of demyelinative disease in man. Current investigations on structure and biological activity of CNS proteins have been oriented around the following major questions: 1) Whether their encephalitogenicity is a characteristic of a multiple component system (i.e., a group of closely related proteins all capable of inducing disease) or is related to a single member of the group. 2) Structural relationships among guinea pig and other species encephalitogens. 3) The relationship between histocompatibility antigens (responsible for tissue graft rejections) and organ specific antigens capable of inducing autoimmune damage. 4) The nature of the specific grouping in purified encephalitogenic protein(s) responsible for development of delayed hypersensitivity and autoimmune damage.

Results of these studies on structure and biological activity can be summarized briefly:

Fractionation of the previously described "purified" encephalitogen coupled with disk gel electrophoresis and bio-assay has led to the conclusion that the fraction consists of one major and several closely related minor components. Encephalitogenic activity was found in at least two but not all of the sub-fractions.

Guinea pig brain basic protein is structurally related but not identical with similar fractions prepared from other species. Basic proteins from other guinea pig organs do not show any immunologic cross reactions with brain basic proteins. Production of inflammatory lesions in heart following the injection of a heart basic protein preparation in Freund's adjuvant was an exciting by-product of this study of organ basic proteins.

Attempts are being made to elucidate the role of circulating antibrain antibodies in experimental allergic encephalomyelitis (EAE)—to ascertain whether they contribute significantly to disease induction, suppression or recovery. Earlier studies have failed to establish

successful correlations because of several obvious defects in the experimental design employed by other investigators. Passive cutaneous anaphylaxis (PCA) has been used as a means of determining one type of antibrain antibody but it has only limited use as a tool in studying the whole antibody spectrum. Other techniques for antibody detection are being developed.

Preliminary to an investigation of *in vitro* protein synthetic mechanisms of sensitized lymphocytes, we have studied the cellular transfer of EAE in inbred guinea pigs. In our hands, the technique works sufficiently well to confirm the published reports of successful cell transfers but not so reproducibly as to make it a useful tool for the purposes planned.

In view of published reports on the possible location of histocompatibility antigens in a membrane lipoprotein fraction, various encephalitogenic fractions were tested and these unique antigens were found to be present in a partially purified bovine cord preparation but not in a more highly purified rabbit cord protein.

These observations on the presence of organ specific antigens and species specific histocompatibility antigens in the same membrane protein fraction are preliminary but are sufficiently important to warrant further detailed investigation.

Section on Psychosomatic Medicine

The Unit on Psychosomatics under Dr. Philippe Cardon has operated in its present form for about ten years. Its goals have been to relate, by clinical observation and experiment, the phenomena of the "psyche" and the "soma." Somatic data are most often indices of circulatory function because of the interest and competence of the responsible investigator, but are often data obtained in collaboration with others. The implicit goal is understanding of ways in which psychosocial adaptions influence general health. Through such understanding it may be possible to define health-promoting modes of psychosocial adaption. Use of such modes by societies may conceivably prove to be an unusually effective form of prophylaxis against many sorts of illness, and furthermore

a form of prophylaxis which persists through generations.

An overview of the work of the past ten years is provided by listing the phenomena which have been reported for the first time as a result of studies in which the Unit has participated: psychic influence on fat mobilization in scurvy; resistance of scorbutic animals to experimental allergic encephalomyelitis; psychic effect of methionine loading in schizophrenic men; dependence of fetal fat content on maternal free fatty acid concentration; *in vitro* stimulation of oxygen consumption of cells by a free fatty acid; influence of cardiac cycle on reaction time; physiological and psychological effects of epinephrine and its metabolism in schizophrenic patients; inverse relation of average morning heart rate to responsiveness to nor-epinephrine; relation of plasma hydrocortisone concentration to age. It will be noted that most of these new observations have to do with hitherto unsuspected or undocumented relationships between spheres of discourse. To date, the literature contains reports of independent attempts to replicate six of these observations, and all confirm the original findings.

Section on Psychiatry

The long-range goals of the Section on Psychiatry under Dr. Jack Durell are related to the clinical research conviction that human behavior must be understood in terms of an interaction between the biological organism and the social field. As a corollary the disturbances in thought, affect and behavior, which constitute the area of interest of clinical psychiatry, need also be understood as an interactive phenomenon. It is the goal of the Section to increase understanding of the mechanisms by which disturbed behavior is generated or dissipated, and this understanding must come through a greater understanding of the interaction between the biological organism and the social field. To implement this goal the resources of the Section have been organized into two psychiatric treatment units operating as therapeutic communities. Patients selected for treatment on these units have major psychiatric disturbances and priority is given to those patients whom one might expect to show major shifts in the clinical state. It is hoped that by

studying patients at times in which major clinical shifts occur, insight can be obtained into the operative biological and social forces. When these shifts occur "spontaneously," they can be viewed as analogous to a natural experiment. When these shifts are drug induced, a specific lever is applied to the biological forces as an independent variable; the mechanism of action of this independent variable is under study, along with the social forces as they respond to the biological changes. The social forces are not as easily controlled as independent variables, but an attempt in this direction is being made in that the two psychiatric units are being constructed with differing milieu philosophies so that the effects of these upon different patients can perhaps be evaluated.

In previous years it had been demonstrated in the therapeutic community for the treatment of schizophrenic patients that marked changes in staff attitude towards patients accompany changes in clinical state of these patients. The follow-up data obtained in a pilot study last year have been partially analyzed and may contribute to our understanding of the relevancy of these observations but the analysis has not been completed and no conclusions have as yet been drawn. A number of instruments are now in use to collect longitudinal data on each patient; these include measures of psychopathology, socio-adaptive behavior and significant events within the families of patients. In addition, extensive social and historical information is being obtained in a formalized manner on all new admissions. Much thought has been devoted to an attempt to conceptualize the forces within each of the therapeutic milieux under investigation so that the suitable measuring instruments can be devised in an attempt to systematize the relationship between these forces and clinical state. Finally, controlled studies have been initiated to which patients meeting a narrow range of criteria for what may be designated sub-acute schizophrenia are admitted. These patients would be expected to have poor prognoses if not treated. The specific hypothesis is that the social *milieu* constructed for the treatment of schizophrenia on one of the units would be most effective for this group of patients. After the initial evaluation of patients and the initial de-

cision that they meet the criteria for the study, they are then sorted at random into three groups; one treated on the unit designed for schizophrenics, a second group treated on the more traditional unit which is now operative within the Section, and a third group returned to the community at large to find treatment as it is available within the community. An extensive follow-up plan has been devised and an effort will be made to find whether there is evidence that either of the therapeutic milieux had any effect on the clinical outcome of the schizophrenic process of the patients studied.

Work has continued this year on an alleged plasma factor in schizophrenics. The work has progressed well and is perhaps nearer a definitive answer to questions regarding the mechanism of action of a substance present in the plasma of certain individuals which affects the metabolism of chicken red blood cells and whether or not this substance is related in any direct way to schizophrenia. The evidence is now quite compelling that the plasma of many humans does exert an effect on chicken erythrocytes and that it exerts this effect through a primary attack on the cell membrane. It has been demonstrated that the chicken erythrocytes have a very marked "Pasteur effect" and that this action upon the cell membrane releases the chicken cell from metabolic control. Further evidence has been strongly supportive of the hypothesis that this effect on the chicken red cell membrane is an immune one and is essentially a complement dependent immune lysis. The population studies have not as yet been completely analyzed but the preliminary analyses are strongly suggestive of the conclusion that the occurrence of this immune phenomena is not more frequent in schizophrenics than in other people who have lived under the same circumstances. Thus, in acute schizophrenia it appears to occur no more frequently than in a control population, and in chronic schizophrenics it appears to occur no more frequently than in other patients chronically hospitalized at the same institutions. It must be stressed, however, that the analysis of the data is not yet complete, and should there be evidence for an increased incidence of this immune factor in a subgroup of schizophrenics, the problem would remain a very interesting

one; it has been suggested from other evidence that there is an abnormality of immune mechanisms in schizophrenia and that this contributes to the disease state. A definitive answer to this possibility is expected within the next few months.

Closely related to the studies of a serum factor in schizophrenia have been studies on the hypothesis that there is an abnormality in the sodium and potassium transport mechanisms in some schizophrenics as evidenced by increased activity of the cation stimulated ATPase in the red cells of these patients. Miss Eileen O'Brien joined our unit last summer to pursue this work, having previously reported findings suggesting that conclusion with Dr. Seeman at the Rockefeller Institute. Assays of enzyme activity on the red cells of a large group of patients were undertaken, and the results make it impossible to rule this hypothesis either out or in. There is some suggestion that certain schizophrenics have unusually high values of this cation stimulated ATPase but the results are not conclusive. This work will be pursued in future years. Also in connection with this work, investigators in the Section are attempting to determine whether they can confirm the presence of elevated macroglobulins in the plasma of schizophrenics as had been reported by Fessel. The results of these studies have not yet been analyzed.

Another major area of biological research relates to the hypothesis that there is an abnormality in catecholamine metabolism within the central nervous system associated with affective disturbances and that imipramine is an effective agent in the treatment of depression because of its effects on catecholamine metabolism. Dr. Schildkraut, before joining the section, had collaborated with a group in Boston which had demonstrated a decrease in VMA excretion in association with imipramine treatment. These results have been confirmed and extended here and, in addition, information has been obtained on the excretion of normetanephrine in relation to imipramine treatment and in relation to recovery from affective disturbances. There are some indications that normetanephrine excretion changes in a characteristic way in relation to the change in the clinical state, but the results will need further

confirmation. If confirmed, they are most interesting since they offer indirect support of the hypothesis that an alteration in catecholamine metabolism is associated with the affective disturbances. It is planned to pursue this hypothesis with several different approaches over the coming years.

A contribution which has been made by the section in previous years relates to the finding that certain catatonic states are accompanied by changes in thyroid function. There had been no really satisfactory prior demonstrations that thyroid function in humans varies in relation to any behavioral or emotional states. Studies on hypothyroid psychoses within the section have also generated hypotheses regarding the possible relationship between the brain stem mechanisms associated with the regulation of thyroid function and those associated with the regulation of affect in behavioral states. Studies during this year have added some clarification to the observed changes in thyroid function in that it has now been clearly demonstrated that in a periodic catatonic patient a peak of thyroid activity preceded the onset of the catatonic episode and that thyroid function dropped off sharply within the first weeks of the catatonic episode.

It is worth noting that considerable staff effort this year was spent on reviewing the literature on biological investigations in psychiatry. This effort proved most valuable in that it has facilitated the focusing of attention on several promising areas of research. The review by Drs. Durell and Schildkraut will be published in the American Handbook of Psychiatry.

Along with the clinically-based work, laboratory investigations are proceeding on a more basic neurochemical level with particular emphasis on membrane function. It appears likely that an understanding of neurochemical events on a basic level depends upon an increased understanding of membrane phenomena; with this increased understanding may come greater comprehension of the biological forces leading to disturbed behavior. In neurochemistry, the major contributions of the section have been the studies of the mechanism of action of acetylcholine on the phospholipid metabolism of brain particulate systems. Evidence has been

gathered that this reaction occurs within the pinched-off nerve endings and that it does not use the adenine nucleotides generated in the media. Evidence has been obtained that adenine nucleotides are synthesized within the pinched-off nerve ending under the conditions of the acetylcholine stimulation. Apparently, however, acetylcholine has no effect on this reaction nor on the permeability of the pinched-off nerve ending to inorganic phosphate. As yet, it has not been possible to demonstrate any intermediates in the stimulated labeling of phosphotidic acids. The hypothesis that the liberation of a peptide might account for the stimulation has led to negative results. The problem remains an interesting one, however, since the possibility that this reaction is related to the mechanism of neurotransmitter function of acetylcholine must be given serious consideration and is therefore of marked basic interest.

CLINICAL NEUROPHARMACOLOGY RESEARCH CENTER

CLINICAL STUDIES

All patients admitted to the William A. White Service since August, 1961, represent the potential if not actual research population. This includes presently about 100 inpatients, over 200 previously hospitalized patients who continue treatment but live and work in the community and, finally, over 300 socially recovered patients who represent the catamnestic study group. Comprehensive clinical and psychosocial research is the *raison d'être* for the multiple clinical facilities and services, consisting of inpatient services, Day Hospital, Clinic, Community Nursing and other home services.

Pending final approval, the Center's clinical operation is now divided into Sections of: (1) Clinical Psychiatry, (2) Experimental Psychiatry, (3) Psychosocial Research. The addition of the Section of Neurochemistry which is currently part of the Laboratory sections was recommended in the draft proposal for the Center's reorganization.

The Center's research potential would seem to be unique, if not unmatched, in view of this combination of research sections, clinical facili-

ties and professional services, if one adds the availability of a clinically and socially widely stratified patient population. The latter can be expected to yield important scientific data within the framework of ongoing and planned investigations.

Insofar as **Clinical Psychiatry** is concerned, investigative interest focuses particularly on rationales for and evaluations of different therapeutic modalities. Explorations of different types of documentation are still in progress to identify and record sets of features believed to be relevant to success or failure of clinical and social therapies. To recognize and distinguish individual characteristics, areas, topics and symptomatologies is also an important educational process which sharpens clinical perception and develops therapeutic conceptualization. This approach has been valuable in shifting attention from generic if not generalizing to individual features of personality, life situations and illness. While we uphold and adhere to principles of nosological diagnoses, we aim at qualification in terms of clinical and psycho-social target symptoms or features for therapeutic and prognostic purposes.

One study with an experimental drug, SKF 20716, was supervised by Dr. von Mendelsohn, who reported the results at the Collegium Internationale Neuro-Psychopharmacologicum, IV International Meeting held in Birmingham, England, in 1964. A pilot study of the feasibility of drug treatment for clinic patients in a group setting was completed by Dr. Post and Dr. Salamon. Their report will be published shortly. Another project by Dr. Salamon and Dr. Post concerned the significance of alpha blocking in schizophrenic patients. While the results are still tentative, questions of method have been clarified and represent a definite contribution to research in this area. The first phase of this project has been completed, and a paper reporting the findings has been accepted for publication. Dr. Richard Veech, whose special interests are in biochemistry, spends part of his time in Dr. Weil-Malherbe's laboratory to study dopamine excretion in Parkinsonian patients and normals and the catecholamine excretion in pinealectomized rats.

Dr. Felix von Mendelsohn resigned on February 1, 1965, to devote himself entirely to

private practice. His many valuable services and extensive clinical skill and knowledge added greatly to the development of the clinical program.

The establishment of the **Section on Experimental Psychiatry** coincided with the appointment of Dr. Kenneth Gaarder on July 1, 1964. Eminently qualified for his assignment, Dr. Gaarder organized the laboratories which are already in operation. The current projects include two main groups: visual evoked response studies, and fine eye movement studies.

Visual evoked responses of three non-normal groups are being systematically collected in three projects. The groups are: psychiatric patients, psychiatric patients on and off phenothiazine medication, and dysleptic drug-treated patients. Studies comparing different recording systems which were carried out while the Laboratory was being set up were reported by Dr. Speck at the Federation Meetings, April, 1965.

Fine eye movement studies would be of academic interest only if "dynamic" theories of vision were completely discarded. However, the finding of evoked responses related to saccades revives dynamic theories. Studies of normal subjects and psychiatric patients are both underway.

The **Section of Psychosocial Research** operates through the medium of clinical social work augmented by research staff on the professional and technical level. The orientation and goals as developed by the Section's Chief, Dr. Mayo, are toward continuity and comprehensiveness of clinical service. Comprehensiveness refers to a unique utilization of social workers. Setting or facility to which they are assigned are secondary to maintaining a therapeutic and investigative relationship with patients and families. Particular emphasis is placed on the social worker's skill and initiative to perform a variety of services ranging from case work, community referrals, job placements and home visiting to systematic psychosocial evaluations. Based on this experience the social worker can develop a truly comprehensive knowledge and concept of the complexity of personality factors, social functioning, family relations and psychopathology. Emphasis is placed on the distinction between social problems arising pri-

marily from external sources and social pathology which reflects internal disturbances.

It is the particular goal of the Section to identify, formulate and investigate specific social psychiatric aspects on a longitudinal basis. Four major studies, mostly still in the formative and data collecting stages, are in progress. The catamnestic study represents a longitudinal assessment of outcome of illness as reflected in social functional patterns of formerly hospitalized patients. Evaluations are based on home visits, interviews of patients, family members and other persons who can contribute significant information. The family study now consists of a nucleus of 132 index cases with an additional 88 index cases which are still being screened. The family study population amounts to approximately 20% of our total patient population. Questions of incidence, race, kinship and social class are of particular interest at this early stage of the study. A poverty study concerned with the influence of poverty on clinical and social rehabilitation is in the planning stage. Our patient population is especially suited for this kind of study since 44% of our patients are Negroes, of which nearly ¾ have been classified as belonging in the socioeconomic classes 4 and 5. Finally, a cultural bias study will have as its aim to determine the role and influence of racial and cultural bias on clinical evaluations. It is well known that misconceptions based on stereotypical assumptions of mentality and behavior can distort clinical evaluations. These in turn tend to limit therapeutic goals. In this study special attention will be focused on the clinician's ability to distinguish between cultural and psychopathological aspects of affect, motivation and social behavior. While these aspects do not lend themselves to objective measurement, they are nonetheless subject to objectively conducted analysis using several observers of different cultural background to establish inter-rater consensus.

The **Section on Psychosocial Research** has been particularly active in the imaginative implementation of new clinical approaches, as well as in the collection of investigative data. Dr. Mayo is also to be commended for an excellent program of training and education for both student and professional social workers.

LABORATORY STUDIES

Section on Neurophysiology

Ongoing studies by this section are directed towards the elucidation of the functional role in the mammalian central nervous system of the endogenous amines acetylcholine (ACh), norepinephrine (NE) and serotonin (5-HT), which have been shown to be present in varying amounts in different brain regions and are suspected of being central neurohumoral transmitters. We are attempting to answer two related questions dealing respectively with the sensitivity to ACh, NE and 5-HT of neurons in various brain regions and with the possibility that unit sensitivity to one or the other of these three substances may reflect a synaptic transmitter function.

In our studies, the endogenous amines and related drugs are ejected electrophoretically from multi-barreled glass micropipette electrodes, directly at the site of extracellular unit recording, to avoid some of the complications introduced by more indirect routes of drug administration. With this method, the major diffusional (e.g. blood-brain) or enzymatic (e.g. circulating catabolic enzymes) barriers are bypassed and the likelihood of confusing indirect for direct effects of the administered substance is reduced.

Our investigations have demonstrated the presence of ACh, NE and 5-HT-sensitive unit in all regions of the brain thus far explored comprising the sensorymotor cortex, the caudate, the lateral geniculate and dentate nuclei, the hippocampus, the hypothalamus, the medulla and the lumbar segments of the spinal cord in cats and the olfactory bulb in rabbits.

In our very first study, some three years ago, we were surprised to find, among ACh-sensitive neurons, an appreciable number of units that exhibited a depression rather than a facilitation by ACh. Later on, we encountered a similar duality of ACh, NE and 5-HT action in several other brain regions and were impressed by the many different unit response patterns elicited by their administration. However, the systematic testing of many thousand neurons in many different brain regions has made clear

to us that what at first appeared as a disquieting array of seemingly unrelated phenomena is in fact amenable to systematization.

The ratio of responsive to unresponsive units for any one of these three substances varies in different brain regions and was found to be influenced by several biological and technical factors. Some of these may have been responsible for an erroneous report by others that, apart from ACh facilitation of Renshaw cells, spinal cord neurons are insensitive to the administration of ACh, NE and 5-HT. On the contrary, they exhibit patterns of responses to these three substances largely indistinguishable from those exhibited by neurons elsewhere in the central nervous system. Such patterns can be classified into two main types, quick or explosive and slow or tonic. Most central neurons exhibit the latter type of response. Renshaw cells were found to yield an explosive response to ACh but a slower, tonic one to NE. Several biological factors can contribute to individual differences in latency of onset and duration of central neuron responses to a given suspected transmitter. These include the size of the neuron, the location and relative concentration of receptive sites on the cell membrane, their distance from the point of drug application, the types and efficiency of local diffusional and enzymatic barriers, the presence or absence of specific or unspecific binding sites on the path of drug molecules, the association and dissociation constants of drug-receptor interactions, the presence or absence of receptive sites of different threshold and opposite function on the same cell, the functional relationship of the tested unit vis a vis other nerve cells in a path of self-sustained reverberating activity, the activation or inactivation of functionally related neighboring units, etc. In view of the multiplicity of these factors, it would be unreasonable to expect each to make an identical contribution in the response of any central neuron to a given substance. On the contrary, much individual variability in speed, direction, duration and other parameters of the response can be expected, as indeed it is found experimentally.

Our evidence shows that a "typical" central neuron response to the suspected transmitters

ACh, NE and 5-HT does not exist, the pattern and direction of the response reflecting individual characteristics of the tested neuron. Regarding the direction of the response it is now certain that each of the three suspected central transmitters can cause either facilitation or depression of unit activity. This became evident when we analysed the response to the three amines of neurons in functionally homogeneous brain populations, that is, neurons clearly identifiable as to their type on the basis of their response to relevant electrophysiological tests. It was found that hippocampal pyramidal and pyramidal tract neurons are consistently facilitated by ACh and, when sensitive to NE and 5-HT, are consistently depressed by these two substances. Mitral cells in the rabbit olfactory bulb, on the other hand, are consistently depressed by NE and also by ACh and 5-HT when sensitive to these substances. On the contrary, Deiters nucleus neurons are consistently facilitated by both ACh and NE.

As we gradually accumulate evidence implicating ACh and NE as transmitters in the hippocampus and the olfactory bulb respectively, it is clear to us that undue reliance upon the neuromuscular junction or the Renshaw cells as the models for central synaptic transmission is both unwarranted and misleading. Similarly, extrapolation of findings from one central cell system to another is unjustified, no two central neuronal systems being presumably identical in all their anatomical biochemical and functional characteristics. Lastly, it is clear to us that while reliance upon pharmacological criteria for central transmitter identification is unavoidable, the evidence thus obtained should be judiciously used since the "specificity" of action of pertinent blockers or potentiators has been demonstrated thus far only at peripheral synapses. Moreover, since these agents differ in their effectiveness and specificity at different peripheral synaptic sites, it may be expected that they will show even greater differences at central synaptic sites. Development and classification of pharmacological tools of proven relevance for the study of central synapses is an essential future step. Meanwhile, progress in the field of central transmitters identification will still have to rely on much hard work,

many different skills and a generous dose of common sense.

Section on Neurochemistry

The Estimation of Catecholamines and Catecholamine Metabolites in Human Urine.

We are continuing our efforts towards the improvement of the methods for the measurement of urinary catecholamines and catecholamine metabolites. When epinephrine and norepinephrine were estimated by the previously described method of optical differentiation (Weil-Malherbe, H., *Z. Klin. Chemie* 2: 161, 1964) results were often unsatisfactory. This difficulty has been traced to various degrees of quenching in some urine extracts, which affected the fluorescence of the epinephrine and norepinephrine derivatives to an unequal degree. For this reason the method of optical differentiation has been abandoned in favor of differentiation by oxidation at different levels of pH. Conditions have been established under which epinephrine is oxidized with only minimal oxidation of norepinephrine and vice versa. The intensity and stability of the fluorescence of the reaction products could be increased considerably by a standardized irradiation procedure. It was found essential to include a series of internal standards and other controls with each determination. We have similarly improved the estimation of dopamine by the method of Carlson and Waldeck (*Acta physiol. Scand.* 44: 293, 1958) which we previously found inapplicable to urine extracts. We have now, however, substituted it for our earlier procedure. Again, the inclusion of internal standards with each estimation was found essential.

During the past year methods for the estimation of metanephrine and normetanephrine in urine have been published by two other laboratories. We have tested these methods but did not find that they offered any advantage over our earlier method. An apparent misprint in one report led to a new procedure for the independent determination of normetanephrine without interference from metanephrine. This modification is still in the stage of development.

The various steps in our own procedure for the estimation of metanephrine and normetanephrine have been carefully checked and revised as regards the time of oxidation and the amount of oxidant required. The fluorescence spectra of the oxidation products suggest that the compounds formed from metanephrine and epinephrine are identical but those from nor-epinephrine and normetanephrine are not.

Our previous attempts at the estimation of 3:4-dihydroxymandelic acid were described in the last Annual Report. They were frustrated by the interference from another closely related urinary metabolite, 3:4-dihydroxyphenylacetic acid. This interference has now been completely eliminated by the use of a highly specific L-mandelic oxidase prepared from *Pseudomonas* fluorescence. This enzyme has previously been recommended for the estimation of vanillyl mandelic acid in urine (Rosano, C. L., *Clin. Chem.* 10: 673, 1964). Preliminary results indicate a concentration of 100–300 µg of dihydroxymandelic acid per gram of urinary creatinine.

During the past year about 650 urine samples were analyzed for catecholamines and catecholamine metabolites in connection with the N. A. S. A. stress project. Some of the results were presented at a conference on the "Military Applicability of Research on Quantitation of Stress by Catecholamine Analysis" held at Beaumont House on January 27 and 28, 1965.

Although not a part of our routine program of urine analysis, a method has been developed for the investigation of neutral and acidic catechol derivatives in urine by two-dimensional thin layer chromatography. The method allows a satisfactory separation of a mixture of 10 authentic compounds likely to occur in urine. Several of these could be identified in urinary extracts, but in addition chromatograms of urinary extracts were sometimes found to contain unidentified spots. Most of these unidentified compounds are apparently non-acidic since they were not retained by a column of anion exchange material.

THE NATURE OF THE "FREE" FRACTION OF TISSUE CATECHOLAMINES. When tissues such as brain or heart are homogenized in isotonic sucrose, the tissue catecholamines are distrib-

uted between a particulate fraction which can be separated by high-speed centrifugation and a "free" or soluble fraction which remains in the supernatant. The coexistence in the intact cell of a "free" fraction of catecholamines and powerful enzymes of catecholamine metabolism is difficult to understand and the following two possibilities are therefore being studied by us: (1) The "free" catecholamine fraction exists in combination with a soluble protein or other macromolecule; (2) the "free" catecholamine fraction is an artefact due to the destruction of a highly labile part of the particulate fraction during the disintegration of the tissue.

The first of these possibilities has been studied by passing high-speed supernatant fractions of tissue extracts through columns of Sephadex G-25 or Bio Gel. Preliminary results showed a clean separation of a protein peak and a catecholamine peak, indicating that the bulk of the catecholamine present in this fraction was not combined with a soluble protein. Further experiments with tissues from animals which have been treated with tritiated norepinephrine are projected.

It is hoped that the second possibility will also be studied by a variation of the conditions of homogenizing the tissue.

STUDIES ON ALDEHYDE DEHYDROGENASE. Little is known about the enzyme or enzymes which oxidizes the aldehydes formed by the activity of monoamine oxidase. Liver and kidney are the only tissues from which active preparations have been obtained although the aldehydes are known to arise and to undergo oxidation in tissues such as brain, heart and spleen. The possibility can not yet be ruled out that the enzyme present in, say, brain differs from the liver enzyme in having a narrower substrate specificity which might be restricted to the aldehydes derived from biogenic amines. The possibility could not be studied hitherto, because the substrates were not readily available in pure form. p-Hydroxyphenylacetaldehyde and homovanillin have now been prepared in our laboratory by a chemical method and their ability to serve as substrates is being investigated. A fraction obtained from bovine liver by ammonium sulfate precipitation has been further fractionated by electrophoresis in polyacrylamide gel and cellulose polyacetate.

Dehydrogenase activity for aliphatic and aromatic aldehydes has been located in a single band which is distinct from that of alcohol dehydrogenase.

STUDIES ON BRAIN PROTEOLIPIDS. An attempt has been made to solubilize in aqueous medium the protein moiety of brain proteolipids. Following removal of most of the lipid by a reported method (Webster and Folch, *Biochim. Biophys. Acta* 49: 399, 1961) the protein was dissolved in anhydrous hydrazine within 30 minutes by means of rapid freezing and thawing. The protein in hydrazine was then chromatographed rapidly on columns of sieving gels (Sephadex or Bio Gel) with aqueous sodium lauryl (dodecyl) sulfate (SDS) or Tween-80 as developer.

A single excluded protein peak was obtained with concentrations of SDS down to 0.003% on Sephadex G-75 and with 0.03% on Sephadex G-200 suggesting aggregation of the protein to a large molecular weight. At 0.1001% SDS, the pattern was more complex. With 0.1% Tween-80, protein was excluded only on Sephadex G-25 but not on the larger pore Sephadex media (G-50 and above) suggesting dissociation to low molecular weight. Turbidity and finally flocculation occurred within a few hours in 0.1% Tween-80 and within days in the case of the SDS. In 0.01% Tween-80, flocculation was more rapid.

More than 70% of the protein is recovered in the excluded peak. Preliminary data suggest that the protein contains bound hydrazine and that part of the arginine and possibly histidine may be modified.

Many other potential solvents, developing solutions or techniques failed to work.

Section on Psychopharmacology

The Section continued its investigation into the mechanism of hallucinogenic drug action using the short-acting alkyltryptamine derivatives as research tools at various levels of inquiry.

METABOLIC STUDIES. At the biochemical level, both the general peripheral metabolism and some aspects of the metabolism in the CNS were given closer attention this year. The quantitative pattern of 6-hydroxylation for several tryptamine derivatives in mice was stud-

ied. N,N-dimethyltryptamine (DMT), N,N-diallyltryptamine (DAT), N-morpholinotryptamine (MOT), N-piperidinotryptamine (PIT), and N,N-diethyltryptamine (DET) were all hydroxylated by the animals *in vivo*, but to different extents. The ranges were very wide, between 6.1% (DMT) and 66.8% (DET), the others being in between on this metabolic measure.

When a new method for separating the monoalkyl- from the dialkyl-6-hydroxy metabolites was developed, and the urine of the animals was tested, we were surprised to find only dialkyl-6-hydroxy metabolites in the urine collected after DMT and DET, but both monoalkyl- and dialkyl-6-hydroxy derivatives after DPT and DAT had been administered. The failure to find 6-hydroxy-monomethyl- or 6-hydroxy-monoethyltryptamine in the urine of animals treated with the corresponding dialkyl derivatives gave rise to the suspicion that this might be due to a possible conversion of the monoalkyl-6-hydroxy derivative into a 7-hydroxy- β carboline structure as suggested by Heinzelman and Szmuszkovicz.

7-Hydroxy-2-methyl- β carboline (7 H²M) a synthetic representative of this type has been made available to us by Dr. Heinzelman and we developed a specific and sensitive quantitative method for measuring this type of compound in biological material. Applying this technique to the urine of mice treated with N,N.-dialkyltryptamine derivatives (DMT, DET, or DPT), the results were essentially negative. Although the hypothesis that β carboline derivatives are formed has not been proven valid, we still cannot explain the differences in the metabolic pattern between DMT and DET on one hand and DPT and DAT on the other. Whether or not this metabolic difference is related to the hallucinogenic action remains to be established in human experiments.

The possible role of hydroxylation in the psychodysleptic action of the highly potent LSD-25 continued to intrigue us. The structure for the phenolic metabolite formed by rat liver microsomes has been suggested to be 13-hydroxy-LSD. Authentic, synthetic compound with this structure was unavailable, but a comparison with authentic 12-hydroxy-LSD-25 showed that our enzymatic metabo-

lite is not identical with this synthetic compound. The two derivatives showed distinct differences in paper chromatography and in color reactions with three reagents.

In an attempt to find this presumably 13-HO-LSD metabolite in the urine of an alcoholic patient who took 500 µg of LSD as part of his treatment program at Spring Grove State Hospital, there was indication that β glucuronidase liberates a fluorescent compound in the postdrug urine with characteristics resembling this metabolite, but too much interfering material prevented definite identification.

Moving closer to the CNS in our metabolic studies, we continued to investigate the interference by these drugs on the serotonin metabolism in small areas of the mouse brain. We used the technique described in last year's annual report but the data collection was slow: some 250 samples from each experiment had to be counted in the Scintillation Counter which took considerable time because of the relatively low activity obtained in some parts of the brain. Nevertheless, the first part of this project was completed with the finding of a specific *in vivo* metabolic effect in small areas of the hypothalamus which we feel could be related to the specific dysleptic effect of the active drugs. To establish the nature of this metabolic effect we are developing a procedure by which we can measure transfer rates between metabolic compartments in small areas of the brain *in vivo*, using a double-labeling technique.

BEHAVIORAL STUDIES. Before attempting to establish the significance of the biochemical findings to the specific psychodysleptic phenomena in man, we need to clarify the relationship of the regional metabolic changes with the behavioral changes which could be observed in animals treated with the drugs.

Some of the most interesting work in the past year was centered around methodological problems involving determination of stimulus generalization gradients and quantification of oscillatory behavior during conflict as behavioral situations potentially sensitive to drugs. E. Hearst has extended some of his earlier findings in the area of stimulus generalization in animals, and has begun some new experiments on this topic. Although in free-responding situations avoidance responses are more likely to

transfer to new stimuli than are approach responses, no large differences in this direction were obtained in a discrete trial situation. Central stimulants like d-amphetamine or caffeine, and hallucinogenic drugs like LSD or mescaline, had an adverse effect on stimulus generalization gradients for avoidance behavior in the monkey; under these drugs monkeys responded more or less indiscriminately to all stimuli, whereas under normal conditions behavior was restricted to certain specific stimuli. Hearst has concluded projects on the effects of stress on well-learned behavior in the rat and has devised some new procedures for quantifying the oscillation and vacillation that is characteristic of behavior under conflict.

With Dr. Hearst's leaving, some of these projects have been terminated but we intend to use those behavior schedules which showed sensitivity to drugs to further clarify the relationship between metabolism and observable behavior.

Dr. L. Faillace and Dr. Y. Filby (who replaced Dr. Hearst) are planning to develop a microcanula-recording electrode combination which can be implanted permanently and could be used to stimulate chemically discreet areas of the brain and record the electrical effect of this stimulation. The influence of this stimulation on specific behavioral schedules (with and without the systemic administration of dysleptic tryptamine derivatives) will be studied in order to find meaningful relationships between regional events in the brain and behavior on one hand, and the effect of dysleptic drugs on these parameters on the other hand.

HUMAN STUDIES. Although the animal studies at the biochemical and behavioral levels might contribute important and valuable clues for the mechanisms involved in the psychodysleptic drug action, the working hypothesis derived from these studies must be validated in human experiments.

A multidisciplinary study is under way (in collaboration with the Clinical Studies Group of CNRC at Saint Elizabeths Hospital) to investigate the metabolic and physiological correlates of psychodysleptic drug action. We are using two short-acting hallucinogens: DET and DPT and a so-called "active placebo", 6-F-DET, which in previous experiments proved a

valuable tool to control the effect of suggestion. It is hoped that we can test the hypotheses on whether or not the intensity of the specific dysleptic action is related to the individual rate of metabolism of these drugs and whether an objective measure of the specific drug effect can be found on computer-aided evaluation of photic response and EEG. The data available so far permits no conclusions at this time.

LABORATORY OF NEUROBIOLOGY

The ultimate goal of the research program of the Laboratory of Neurobiology is to elucidate physico-chemical bases for various physiological and behavioral processes taking place in the nervous system. The research activity of this laboratory can be divided into the following four general categories: (1) studies of excitable membranes, (2) investigations of physiological properties of neuroglial cells, (3) analysis of the electric activities of the cerebral cortex of waking animals, and (4) studies of sensory mechanisms. Diverse but well coordinated work is needed in order to be able to make progress toward achieving the ultimate goal.

During the fiscal year 1965, considerable progress has been made in the field of physico-chemical studies of the excitable membrane. By perfusing the interior of the squid giant axon with various proteolytic enzymes, it was shown that the protein molecules in the membrane play an essential role in the process of action potential production. The effects of various intracellular anions and cations were shown to be determined by the affinity of these ions to the oppositely charged groups present in the membrane macromolecules. Further experimental evidence was obtained in support of the "two stable state hypothesis" of nerve excitation. It was suggested that transition of the membrane from the resting state to the active state represents a sudden change (first order phase-transition) of the membrane macromolecules.

The project on neuroglia was pursued efficiently by Drs. F. Walker, I. Singer and T. Takenaka. By using both tissue culture material and cells acutely excised from young guinea pigs, it was shown that neuroglial cells exhibit rhythmical changes in their membrane resistance and potential. Furthermore, it was dem-

onstrated that these cells tend to migrate in the field of electric current. Also, it was shown that neuroglial cells are highly sensitive to various neutral salts and pharmacological agents (e.g., strychnine, choline, etc.). This finding offers a new basis for interpreting the effects of various drugs on the nervous system. Attempts are now being made to demonstrate electric and chemical interactions between neuroglia and neurons.

The electrical activity of the cat cerebral cortex was studied by Dr. E. Podvoll with multiple recording electrodes chronically implanted at many levels of the auditory system. A gradual decline in transient electrical activity has been demonstrated in response to repeated presentation of auditory stimuli, but only when the acoustic stimuli decreased in its ability to arouse the animal. There was a significant difference between the transient and sustained components of electrical activity in relation to repeated acoustic stimulation. The transient component was found to be more directly related to the "novelty" of the stimulus.

Dr. Podvoll is continuing his effort to construct a three dimensional current vector representation of electrical activity in the auditory cortex in response to acoustic stimulation. Unique current vector patterns are being mapped out in both deep and superficial layers of the cortex and in the underlying white matter. It was shown by this method that invasion of nerve impulses into the auditory cortex generated electric currents which change their direction, as well as their intensity, as functions of time.

Dr. Galin's experiments on central control of sensory input in waking cats has been extended to include positive as well as negative reinforcement, monitoring of autonomic indicators of arousal and differential conditioning procedures. These experiments are expected to show whether the changes in auditory activity observed so far are nonspecific effects of anxiety and attentiveness or represent selective changes related to the meaning of specific stimuli.

A technique has been devised by Dr. A. Lansky to perform electrical recordings and measure ion fluxes across a membrane formed by the pigment epithelium of the retina plus

the choroid. By using this method it was demonstrated that: (1) a d.c. potential can be recorded across the isolated pigment layers of the eye, which suggests that a sizeable fraction of the resting potential across the retina originates outside the pars optica, and (2) a major part of the short-circuit current is carried by chloride. Considering the embryological and morphological features of the pigment epithelium it is hoped that the information provided by this preparation will help to identify some of the functional properties of ependymal cells and perhaps of neuroglial cells in general.

It was shown by Mrs. R. Marimont that the assimilation effect in visual perception of contrast (described by Helson), whereby a gray background of narrow white stripes can look lighter than a gray background of narrow dark stripes, can be explained by retinal inhibition, or its equivalent, local averaging, in combination with the blurring produced by the optical imperfections of the eye.

The SAAM (formerly NIH-OMR 9B) computer was used to extend the Fuortes-Hodgkin model for the visual response of the *Limulus* eye. The nature of the gain control was improved by adding a second feedback loop which gave more realistic long-term behavior, and changing the source of the first feedback loop. The possibility that feedback may be controlled by all rather than one stage is being explored.

MENTAL HEALTH STUDY CENTER

Introduction

The Mental Health Study Center was established by the National Institute of Mental Health in 1948 as an all-purpose mental health demonstration clinic; its location, Prince George's County, Maryland, has continued over the years as the site of its operations. During the late fifties and the early part of the present decade, the Center gradually intensified its efforts in terms of evaluation, operational research, and program planning while continuing to provide some outpatient services to county residents and consultation services to local agencies and professionals. The period from 1961 to the present reporting year (1964-65) has been noted for the increased emphasis on

research, the growth in size of the Center's staff, and the addition to the professional staff of several behavioral scientists. This emphasis is reflected in the number of research projects concerned with community social structure, special population groups, and studies of mental health resources and their recipients. The present diversified program of community mental health research, clinical activities, consultation services, program planning, and evaluation conducted in a local community calls for a high degree of cooperation from community agencies as well as from professional and lay groups for the securing of data and for the successful implementation of program.

In carrying out its objectives, the Study Center has been administratively organized into Sections each having a Chief along with professional and supporting staff to accomplish discrete program responsibilities relating to clinical activities, community studies, and studies of adolescence. The Sections are seen as complementing the administrative and professional leadership of the Office of the Director. This report reflects the summary of activities as authored by the Sections with minimal editorial changes. Such an approach, it is felt, provides opportunity not only to cite accomplishments but also to flavor philosophy with aspirations and anticipations of future work by the very people engaged in the essential program elements of the Center.

Office of the Director

In presenting the Center's work for the year there exists the understandable temptation to point with pride to the scope of activities undertaken, to draw attention to program progress, to cite project accomplishments, and to look toward future attainments with realistic optimism. The true essence of these phrases, however, calls for recognition of the many people who contribute over time to the organization's work. That all may share in pardonable organizational pride, special expressions of appreciation are due at this time to the Section Chiefs for their assistance to the Acting Director in maintaining continuity of program following the January transfer of the former

Director, Dr. James Osberg, to his present position as Chief, Community Services and Research Branch, to all senior professional staff for their dedication to the tasks at hand particularly at a time of uncertainty regarding future Center direction, to the Librarian and her assistant for critically needed resource help, to all research assistants and social science analysts for their high quality contributions to the many projects and studies, to the Administrative Officer and assisting personnel for their guidance and counsel in the many administrative matters attendant upon program operation, and to all secretarial personnel not only for their fulfillment of myriad tasks but also for their very human contributions to the spirit of the organization.

While the Office of the Director contains central services and administrative responsibility for the total program, professional staff also became actively involved in significant program planning efforts at the County level. Illustrative of these activities are the following:

Comprehensive Mental Health Planning

The Study Center has continued to maintain its commitments in the area of comprehensive mental health planning within Prince George's County. Mental health planning represents a community-based effort to gather, through the support and assistance of concerned individuals and varied organizations within the community, basic information about existing resources and present and future needs. It has seemed appropriate, then, that the Center's involvement be expressed by individual staff members acting in advisory and consultant capacities to community leaders and organizations.

The Advisory Board of the Center took the initiative in planning by constituting itself as the nucleus for an *ad hoc* committee for mental health planning within the County. Additional individuals representing various organizations within the County were invited to join to make the membership of the group consonant with that of the State Planning Committee. The expanded *ad hoc* committee was recognized by the County Health Officer and later officially

designated by the County Commissioners as the Prince George's County Mental Health Planning Committee.

Dr. Osberg, former Director, Mr. Rooney, Acting Director, and Dr. John Hartley, Anthropologist, have functioned in advisory and consultant capacities to the Committee. In addition, Dr. Hartley has been the technical consultant to the Subcommittee on the Clergy, charged with surveying the clergy as a mental health resource within the County.

During the past year, the Planning Committee has carried out the mental health survey of existing resources and needs following the guidelines and format designed by the State Planning Office. In addition, the local committee has supplemented the State guide with a brief county questionnaire. A report of findings and recommendations for future program action is being sent along to the State office. In addition, strategies are being devised for interpretation to the broader community of the significant findings of the report.

Since Prince George's County is also one of twenty-two communities across the country engaged in a study of total health needs as part of the program of the National Commission on Community Health Services, there is the opportunity to observe first-hand in a local setting the impact of simultaneous studies and to note the necessary steps required to insure cooperative but nonduplicative efforts. For the Center, this involvement in program planning affords an excellent opportunity to contribute as a partner in local endeavors and to note the inter-relationships of planning between state and county programs.

The Center's Advisory Board

In addition to its role as stimulator for county comprehensive mental health planning, the Advisory Board has continued as a major bridge between the local community and the Center. Composed of representatives from various health, educational, social welfare, religious and civic organizations in the County, the Board is a derivative group of the original 'founding fathers' of the Center. With rotating membership consisting of three year maximum terms, this Board has continued in an advisory

and liaison capacity throughout the entire history of the Center's presence in the County. Through its collective action, the Board has served to interpret the Center's work throughout the years; by its individual membership, the Board has frequently been the necessary contact point for initiation of major projects; this has been particularly manifest in the leadership and sanctioning role provided by the clergy representative in aiding the Center in conducting a clergy survey in the County. Additionally, relationships formed between staff and board representatives have an enduring quality beyond term of board membership providing for informed county leadership in mental health and for continuing significant community relationships necessary for program operation.

Clinical Study Section

The preparation of this report marks the end of the fourth year of the operation of the Clinical Study Section. During this time the program of the Section has evolved from that of a clinical operation providing mental health services to the community to include a program of clinical research and community mental health training. During the first two years of operation, clinical service, consultation, and community demonstration were paramount; in the third year many research projects were initiated, and during the past year there has been a continuation and culmination of research projects and an initial effort to think through the requirements necessary to provide a high quality work-training experience in community mental health for career development officers in psychiatry.

The professional staff of the Section consists of the Section Chief, a research psychologist, three psychiatric social workers, a mental health nurse consultant, and supporting staff. In addition, two psychiatrists in the Career Development Program are presently assigned to the Section for a two year period as part of their career development training experience. The time of the professional personnel of the Section has been spent in four general areas: administration, service oriented activities, clinical studies, and training.

Administration

During the past year, a major effort to re-assess program goals of the Section has been underway. Several factors contributed to this need, particularly the discontinuity resulting from three changes in the Section Chief in three years time, and problems inherent in attempting to change the identity and function of a Section from a demonstration and service orientation to an action research orientation. Operationally, such a change means that the Section cannot be quite as free to respond to some requests from the community for service, but must respond selectively in areas that are particularly likely to reward research effort with results which would then enhance the service in a particular area. For example, clinical research efforts to develop effective short-term means of approaching problems of whole families before catastrophic breakdown has occurred requires major investments of time, resulting in less time for the research and treatment of problems that have already reached a stage of severe deterioration. The project "Multiple Family Group Therapy" is an illustration of such an effort to devise a means of approach to the family via the moderately disturbed adolescent, with the hope that such a short-term, focused, effort made with a group of families with common problems might serve as a measure of secondary prevention.

The fact that historically the Section has attempted to respond to requests for service and consultation in widely divergent areas has resulted in a diffuseness in research and consultation activities. This is an issue that is not only of concern to us, but should be a concern of any mental health center performing research and service. The question is whether a small unit can perform truly expert consultation and research around a host of areas or whether it would be best to focus consultation and clinical research activities around specific population groups or specific problem areas. The issues involved are complex and important, and the Section Chief is presently collaborating with the Director of Consultation Activities and the Section's Research Psychologist in considering them.

In the area of clinical studies, there has been a gradual and natural development in the Section of an interest in the problems of adolescence with particular emphasis on such considerations as the study of various short-term therapeutic approaches to adolescent problems and the roles the parents and siblings should play in the therapy. Several treatment techniques are available: conjoint family therapy, collaborative therapy with parents and adolescents, adolescent groups and parent groups, as well as an experimental approach under investigation by this Section, multiple family group therapy. If one's theoretical orientation is that the person identified as the patient is, in many cases, merely the presenting symptom of a pathological family process, then there is an obvious need for ways to scientifically describe the dynamics and pathological processes of the family and its interaction. It is also essential that we strive to define specifically the technique of intervention used by the therapist to alter such pathological processes and that we develop reliable and clinically meaningful methods of objectively assessing change in the family process. Considerable attention is being given in this Section to these problems in the form of formal and pilot research projects.

Another area which has required considerable thought is the development of a high quality work-training experience in community mental health for career psychiatrists in the Public Health Service. Currently, an effort is being made to expand and revise aspects of the clinical program to assure a rewarding experience to the career officers to be assigned here in the future. Obviously, this interim two year work-training experience between the more formal professionally required training of residency and the later assignment to administrative responsibilities requires careful consideration of the ingredients which will best prepare the officer to carry program responsibility in the future.

Service Oriented Activities

A. DIRECT PATIENT SERVICES. Priority of acceptance of cases for clinical service is given to those related to consultation programs, research projects, and specific training activities.

Another group having priority for acceptance for direct service are those community caretakers who are seeking psychotherapy for themselves. This is done for the obvious reason that such help will hopefully have the secondary benefit of improving whatever mental health role they play in the community.

Direct patient services provided include diagnostic evaluation, psychotherapy when indicated, or referral to other resources. Various therapeutic procedures are used including individual psychotherapy, group psychotherapy, family therapy, drug therapy, and multiple family group therapy. Statistical reports of patient activities are included in Tables 1, 2, 3, 4, and 5. These reports are based on cases terminated during the period July 1963 through June 1964.

B. CONSULTATION SERVICES. Historically, consultation services have been offered as part of the operation of the demonstration community mental health clinic. Subsequently, the consultation program provided the opportunity to study the consultation relationship and the evaluation of consultation. The consultation program consists of service to the following:

1. Family Service Agency. Regular individual case and group therapy consultation has been provided to the social work staff of Prince George's County Family Service Agency.

2. Pupil Personnel Department, Board of Education. The Clinical Study Section has provided consultation to the Pupil Personnel Department for many years. The pupil personnel worker plays a key role in the public school system, for it is his responsibility to assist in the management of all school children who have either mental or physical disabilities requiring assistance beyond what is available in the classroom. They represent the school to the family and attempt to get the family to look realistically at the problems confronting the child and to take appropriate action. Since mental disability of a psychological nature is very often related to overall psychological disturbance in the family, it is obvious that the pupil personnel workers' role requires considerable skill. The Section staff provides consultation in two different ways. One member of the Section meets regularly with a group of pupil personnel workers. Discussion of specific

problems is used as a jumping off place for general discussions of the techniques of interviewing, guidance, selection of most appropriate referral agency, and the problems related to making a referral in the best possible way. Individual case consultation is also provided to pupil personnel workers at their request.

3. Parent Education Program (Board of Education). The Clinical Study Section continues to provide consultation to the parent education program which is held under the auspices of the Coordinator of the Child Study Program, Prince George's County Board of Education. The program consists of 21 parent discussion groups distributed throughout the County which meet regularly during the school year to discuss general matters of interest in family life but with emphasis on learning about normal child growth and development. While the groups are not organized to discuss and deal with specific problems of child rearing, discussion of normal growth and development obviously include discussion about the needs and drives of the child in each phase of development with some consideration being given to how parents and others may play a facilitating or hindering role. This is by far the most active family life education program in the County and is probably the most important from the standpoint of primary prevention. As mentioned, although the function of the group is not to be primarily problem-centered, it is suspected that a number of parents enter the group seeking answers to specific problems already occurring. Important aspects of the Section's consultation with the Coordinator of the program include problems of selection of leaders, problems related to both the content and process of training of leaders, a consideration of what are the best methods of running such a group in terms of both group process and content, and problems related to handling specific problems of participants in the group.

4. Nurses and Attendants of Psychiatric Wing, Prince George's County Hospital. This consultation program began this year growing naturally from the incorporation into our program of a provision for Career Development Officers assigned to the Clinical Study Section to spend one month per year on the inpatient

psychiatric service of the County hospital. This is a 20 bed psychiatric wing which is presently being expanded to 50 beds which provides psychiatric treatment to those inpatients who are thought likely to be able to return to the community within a short-term period, approximately 30 days. The nurses and attendants on the psychiatric wing felt a need for the opportunity to regularly discuss general problems of ward management and some instruction to enhance their understanding of the dynamics of psychiatric conditions, particularly insofar as this would aid them in their relationships with the patients. A Career Development Psychiatrist who has firsthand familiarity with the setting, having rotated through the inpatient service, accepted the invitation to provide regular consultations about these matters.

5. On-call Consultation to Other Community Caretakers, such as: County Physicians, Clergy, Public Health Nurses, Probation Officers, and Social Workers from Vocational Rehabilitation, Welfare Department, etc. The Section staff continues to provide on-call consultation service to general community caretakers. These consultations range from telephone conversations with the caretaker about his clients to seeing the client here for evaluation, after which, if appropriate, the client is referred back to the caretaker and further discussions held with the caretaker about the role he can play in alleviating the problem.

C. COMMUNITY AND NATIONAL ACTIVITIES AND PROGRAM DEVELOPMENT. The professional staff of the Clinical Study Section has long felt that it was not enough to provide clinic service and consultation service to the community, but that perhaps greater impact in the long run could be made by their active participation in community agencies which have as their primary responsibility improvement of the health of the community. Thus, professional staff of the Clinical Study Section serves as members of the following boards:

1. Prince George's County Council on Alcoholism
2. Advisory Board, Parent Education Program (Board of Education)
3. Advisory Board and Case Committee, Family Service Agency of Prince George's County

4. Advisory Board of Prince George's County Day Care Center for Retarded Children
5. Advisory Board for the Prince George's County Bureau of Mental Health School Consultation Program
6. Health and Welfare Council, National Capital Area
7. Mental Health Sub-Committee of Public Health Advisory Committee of District of Columbia
8. The Community Health Study Committee's Sub-Committee on "Health Needs of the School Age Child (5 to 19 years)"

Such involvement in local groups provides the opportunity not only to keep mutually informed but has provided the opportunity for consideration of the County's needs as a whole and some discussion of what is available and what is needed to provide comprehensive coverage, and an opportunity to consider what role each agency and group might play.

In addition to participation in local groups, the professional staff participates in non-local workshops, TAP's, etc. For example, the mental health nurse consultant in this Section recently served as consultant to a County Health Department of North Carolina which was planning a follow up program for patients hospitalized in the state hospital, an area in which the nurse had considerable personal experience. Another example is the workshop entitled, "Mental Health Planning for Pediatric Hospitalization" which was initiated, organized, and chaired by the Section's Research Psychologist at the March meeting of the American Orthopsychiatric Association.

Clinical Studies

From the discussion above of the various patient and consultation services, it is easy to see that many research questions would naturally arise. The emphasis in modern psychiatry on the study of the family and the environment in interaction with the individual, and the influence each has on the other, cries out for studies of family process, the consultation process, the methodology of the study of the nature of psychological change in the individual and

groups, etc. Below is a brief narrative statement about the present stage of the projects of the Clinical Study Section. The natural aspect of the origin of the questions investigated is sufficiently obvious without connecting statements. That is, the research projects arise out of the functioning of the mental health clinic. In those instances where projects have culminated in publications or have been accepted for publication, less comment will be made since the results will be readily available.

A. MULTIPLE FAMILY GROUP THERAPY. This is a beginning effort to devise and evaluate various short-term treatment approaches. It was hypothesized that a multiple family group composition would incorporate advantages of both traditional group therapy and family group therapy. Four groups with the composition of three family groups each have been studied. The identified patients in three of the groups consisted of gifted adolescent underachievers. The identified patients in the fourth consisted of juvenile delinquents. Results of the pilot phase of this endeavor were reported at the American Group Psychotherapy Association, January 1965, and will be submitted for publication to the International Journal of Group Psychotherapy. An abstract of the project is scheduled to appear in *Psychiatric Spectator*, April 1965.

B. FAMILY CLASSIFICATION PROJECT. This project consists of an attempt to devise an instrument to assess and describe in quantitative fashion the degree of latitude in decision making within a family along an authoritarian-equalitarian axis, and to assess the appropriateness of the decision. It also attempts to describe the style of family interaction. Reliability studies are complete and comparative data is being collected on delinquent, underachieving, and normal adolescents and their families.

C. EVALUATION OF THE PHYSICAL EXAMINATION AS PART OF PSYCHIATRIC CLINIC INTAKE PRACTICE. This study attempted to ascertain whether the requirement of a physical examination as part of the intake procedure of a mental health clinic interfered with patients' use of clinic facilities. Other questions related to the frequency of important medical findings in the intake physical examination and whether hav-

ing such a policy enhanced the relationship between mental health clinic staff and local physicians. Results of the study have been accepted for publication in the American Journal of Psychiatry and the Maryland State Medical Journal.

D. AN EVALUATION OF THE OUTCOME OF REFERRALS TO A MENTAL HEALTH CLINIC. An evaluation and discussion of the effect of clinic intake policies on the service provided to the patients and community caretakers has been accepted for publication in Social Work, April 1965.

E. A SURVEY OF THE MENTAL HEALTH ACTIVITIES OF GENERAL PRACTITIONERS AND MEDICAL INTERNISTS. The Physicians Survey of Prince George's County is a joint undertaking of the Prince George's County Health Department, the Mental Health Study Center, and the Biometrics Branch of NIMH. The survey was designed to determine the proportion of patients seen by general physicians and internists in the County that are felt by them to have psychiatric problems. The survey has been completed, with 75% of the physicians participating. We received 7,814 reports on patients, and seven percent were considered to have a psychiatric problem. A preliminary report of an analysis of these data was presented to the Mental Health Steering Committee of Prince George's County at the Health Department on January 14, 1965. A report is to be submitted to the Journal of the American Medical Association.

F. AN EXPLORATION OF MENTAL HEALTH ROLES OF PUBLIC HEALTH NURSES: A STUDY OF OCCUPATIONAL CONTINGENCIES AFFECTING MENTAL HEALTH PROGRAMMING. This is an occupational study which attempts to determine what the present mental health roles and attitudes of Public Health Nurses in Prince George's County are in their day-to-day functioning and an evaluation of the effect of short-term work experience in a mental health clinic on these roles and attitudes. Exploratory interviews and field observations with the Public Health Nurses and collection of supervisors' questionnaires as well as exploratory interviews with Mental Health Bureau personnel are completed. At present a series of observa-

tions, interviews, and consultations around the training of Public Health Nurses who are being rotated through the Bureau of Mental Health is underway.

G. THE NATURE OF THERAPEUTIC CHANGE IN ADOLESCENT DELINQUENT BOYS. This was an evaluation of the nature of personality change resulting from an experimental psychotherapeutic intervention with adolescent delinquent boys. It was found that this intervention produced marked behavioral change. Psychological evaluations were done at intervals during the course of therapy. The results have been analyzed along many dimensions to determine whether the sequence of change that was predicted by psychoanalytic ego psychology actually occurred. Although a control group of non-treated delinquents was used initially, answers to certain theoretical questions require the study of a normal control group using the same psychological instruments over the same period of time. Such a group is now being studied. A three year follow-up is planned for all groups.

H. A COMPARISON OF TWO GROUPS OF MENTAL PATIENTS FROM PRINCE GEORGE'S COUNTY, MARYLAND, ADMITTED TO TWO DIFFERENT TYPES OF HOSPITALS (GENERAL AND STATE) WITH SPECIFIC EMPHASIS ON THE DECISION MAKING PROCESS. The role of the family in the decision to send a member to the psychiatric ward of the general hospital or to the state hospital is currently being studied. The social and demographic factors of the two groups of patients will be compared. In addition, the attitudes about mental illness, the uses made of community resources, the previous experience with these and other mental hospitals are being considered. Data collection and coding are being done concurrently and the data will be processed in the near future.

I. ANALYSIS OF TELEPHONE INQUIRIES MADE TO THE INFORMATION SERVICE OF THE MENTAL HEALTH STUDY CENTER DURING CALENDAR YEARS 1962 AND 1963. Telephone inquiries made to the Mental Health Study Center during the calendar years 1962 and 1963 are being analyzed along the following dimensions: the day of the week and time of the call, the family member who makes the call, the person

called about, census tract data regarding socio-economic status, the referral source, the nature of the problem, disposition, and the effect which the call has on the family's subsequent use of mental health resources. Coding is nearing completion and data will be processed shortly.

J. FIVE MARITAL COUPLES IN GROUP PSYCHOTHERAPY: SELECTED TRANSCRIPTS OF GROUP INTERACTION FOR USE IN TRAINING GROUP PSYCHOTHERAPISTS. Material from tape recordings of group meetings was selected and a manuscript prepared which illustrates some of the dimensions of group dynamics and group psychotherapy. It should prove useful for the purpose of setting the stage for discussion of various group issues; hence, should be useful for training purposes.

K. AN ASSESSMENT OF CERTAIN ASPECTS OF CONSULTATION PRACTICE. The purpose of this study is that of assessing certain aspects of mental health consultation from the viewpoint of both the consultant and consultee, and to explore the relationship, if any, between perceived success of consultation, and congruity of viewpoints relative to helpful and non-helpful aspects of consultation.

L. A STUDY OF CERTAIN SOCIAL PSYCHOLOGICAL CHARACTERISTICS OF RECIPIENTS OF A PARENT DISCUSSION GROUP PROGRAM. This study is an attempt to collect basic information regarding the recipients of a major family life educational program in Prince George's County. Collection of data is through questionnaires completed at the beginning and end of a program. Follow-up of dropouts from the program is currently underway in order to have a means of comparing their characteristics with those parents who complete the program.

M. MEANINGS ASSOCIATED WITH CONCEPTS RELATED TO MENTAL ILLNESS: A STUDY OF FOUR DIFFERENT PROFESSIONAL GROUPS IN A COMMUNITY. This is an attempt to devise an instrument which will then be used to assess mental health attitudes of various groups of community caretakers. A modified version of the Osgood Semantic differential scale is currently being used with groups of police, school counselors, welfare workers and personnel in

mental health clinics. Comparison between these groups will be done with educational background and experience with defined mental patients being considered as variables. This study is potentially useful to the staffs of community clinics endeavoring to set up consultation programs with community caretakers since it provides a means of assessing current viewpoints of the potential consultees.

N. PERIODIC SURVEY OF OUT-PATIENT MENTAL SERVICES AVAILABLE TO PRINCE GEORGE'S COUNTY RESIDENTS. A quarterly survey of mental health services in Prince George's County is underway. The survey has been useful in providing comprehensive information to County agency personnel about treatment resources and has resulted in more expeditious clinical service.

O. A TEN YEAR SURVEY OF MENTAL HEALTH PROBLEMS AS DEFINED BY COMMUNITY RESOURCES. This is a review of the problems that this community clinic was contacted about over a ten year period. It will provide information about the different perspectives of community caretakers and agencies about mental health problems, and will also provide data about the characteristics of individuals accepted for service who did not follow through.

P. TRICHOTILLOMANIA IN CHILDREN. The referral to the clinic in the past few years of several children with trichotillomania has resulted in an effort to study the problem in children through the combination of clinical research and literature review.

Training

The Clinical Study Section is pleased to have the opportunity to have Commissioned Officers in the U.S. Public Health Service who are included in the Career Development Program for Psychiatrists assigned here for a period of two years immediately following their residency in psychiatry. Considerable thought has been given and is being given to the qualities, experiences, and knowledge they will need in their future roles in the Public Health Service. At the present time we attempt to provide the following:

A. A SETTING FOR THE DEVELOPMENT OF PROFESSIONAL MATURITY. The Career Officer

in psychiatry comes to the Study Center immediately after residency. At this point, we feel that previous roles of pupil and teacher should be discarded. The pupil and teacher have become colleagues, and there should be mutual teaching and learning. Without this development of a peer relationship with his professional colleagues in which there is mutual respect and guidance instead of instruction, the psychiatrist will never achieve the confidence that will be required of him in an administrative leadership capacity.

B. A SETTING THAT PROVIDES FOR PROFESSIONAL GROWTH IN ONE'S OWN PROFESSION. The Career Officer has learned the basic skills in psychiatry and it is important that he continue to practice them. The Clinical Study Section provides him with the opportunity to continue clinical work in the traditional methods of individual, group, and family psychotherapy. He sees a variety of cases for diagnosis, and he has the opportunity to participate in the development of experimental approaches to patient care. Available among the Section's staff are experienced people of all disciplines who can serve as consultants to him. In addition to these persons, outstanding practitioners of particular therapeutic approaches are brought to the Study Center regularly as consultants who are available for longitudinal case supervision. In addition to the specific clinical work, the Clinical Study Section provides an atmosphere in which both the administration and the staff are intellectually vigorous, and appreciate and encourage intellectual vitality and growth. Knowing that it is important to provide for such in the formal structure of the program, the Career Officer is provided the opportunity to hear guest speakers in a scientific colloquium series, and can participate in a journal club which attempts to sample the mental health literature. In addition to the above, the Career Officer can, if he wishes, spend one-half day a week in a non-paid affiliation with a department of psychiatry of one of the nearby medical schools.

C. A SETTING THAT PROVIDES FOR THE DEVELOPMENT OF A PERSPECTIVE OF PSYCHIATRY'S POSITION IN TIME. The Clinical Study Section provides the Career Officer with the opportu-

nity to engage either cooperatively or independently in clinical research projects. It is our conviction that it is rare that a person will develop a perspective of where psychiatry is and what steps it might next take without some personal involvement in psychiatric research. This involvement would require that he think through what at least some of the next steps might be and also would have the added advantage of bringing about understanding of methodological considerations in research, since he would also have to ask the pertinent questions about design and methodology.

D. A SETTING THAT PROVIDES FOR THE DEVELOPMENT OF A SOUND PERSPECTIVE OF WHAT RELATED DISCIPLINES HAVE TO CONTRIBUTE TO A SCIENCE OF HUMAN BEHAVIOR. This is of major importance. It is our conviction that it is unlikely that any such understanding will come about without the provision of the opportunity to "rub elbows" with *outstanding* spokesmen of other mental health disciplines. These should include not only clinical psychology and psychiatric social work, but also sociology, anthropology, and social psychology. Articulate spokesmen of all these disciplines are a part of the staff of the Mental Health Study Center and work alongside and with the Career Officer during his assignment here. When there are mutual interests, the Career Officer can participate in research projects that are ongoing in the other sections of the Mental Health Study Center.

E. A SETTING THAT PROVIDES FOR THE DEVELOPMENT OF AN UNDERSTANDING OF COMMUNITY FORCES. The development of an awareness of who does what in a community in the area of mental health and how they see their problems and how they think about the solutions to them is a vital aspect of the experience of any person who is going to have certain types of psychiatric responsibility. It is our conviction that no such understanding will come about without direct involvement in a working relationship with many of such people. The Clinical Study Section provides the opportunity to the Career Officer to gain first-hand familiarity with a variety of personnel and community agencies who attempt to deal with

the emotional problems of the population at the local level. Included are the school counselors, school nurses, principals, pupil personnel workers, the probation officers, welfare workers, public health nurses, local physicians and clergy as well as the family agencies, vocational rehabilitation, county hospital, the county health department facilities for the mentally ill and the local societies that are concerned with the problems of mental health. The working contact with this group is via the channel of consultation to them. In such a relationship both gain. The psychiatrist contributes to the knowledge of the community agent through a discussion of the psychodynamics of the problems of individuals or families under discussion, and the county agent contributes to the psychiatrist's knowledge of the community, its problems, and its present methods of handling them. In addition to case consultation with a variety of community agents, the Career Officer has the opportunity to work for a brief period of a few weeks in a local hospital and/or county health department if he so wishes. In this capacity, he contributes by helping get pressing clinical work done, with the result that the physician in charge makes himself available to discuss the problems of administration, how he thinks about these problems and the plans he has for dealing with them.

F. A SETTING THAT PROVIDES FOR THE DEVELOPMENT OF JUDGMENT IN THE PLANNING OF PROGRAMS, AN AWARENESS OF THE COSTS OF SUCH PROGRAMS, AND JUDGMENT IN THE DELEGATION OF RESPONSIBILITY. As mentioned above, the Career Officer has the opportunity to get to know persons in the community who have administrative responsibility for the planning of programs, and learns firsthand how they think about planning. Also, he has the opportunity to participate in the discussions concerning the planning of programs of the Mental Health Study Center. It is necessary for a person who will have responsibility in the future for the planning of sound programs to overcome unrealistic notions that he might have as to how programs really develop. To see and feel the effort that goes into the conceptualization and evolution of research projects and clinical programs is essential.

In addition, the Clinical Study Section has provided on a regular basis a group therapy seminar, consultation seminar, family therapy seminar, and a child psychiatry seminar. A colloquium series of outside speakers is also available to the Study Center as a whole. Through these formally structured activities there is ample food for thought for the stimulation of growth in one's professional discipline.

Community Projects Section

The Community Projects Section was established in 1961 with Dr. James G. Kelly as Chief. After Dr. Kelly's departure for Ohio State University on September 9, 1964, Dr. J. R. Newbrough became Chief of the Section. It has developed over the past four years into a laboratory for the study of the complex interrelationships between the functioning of individuals and the social environment. The normal individual in his naturally occurring social groups as well as the mentally ill and the social institutions designed specifically to deal with them are the foci for our inquiries. The Section staff has developed a series of projects that deal with different aspects of individual behavior and group organization and that relate to each other through shared data sources and shared interests of the investigators.

The research efforts within the Community Projects Section are grouped in the following way in the subsequent discussion, although most of the studies are relevant to more than one category:

1. Community social organization
 - a. Studies of naturally occurring social groups and institutions,
 - b. Studies of the social and psychological correlates of residence in metropolitan subareas.
 - c. Studies of social institutions specifically dealing with the treatment and control of mental disorder and behavioral deviance.
2. Individual and group behavior
 - a. Studies of personality and social characteristics in the normal population,

- b. Studies of personality and social characteristics of deviant individuals and groups.

Community Social Organization

Prince George's County, Maryland is the geographic area toward which the primary work of the Section, and of the Study Center, is directed. It has a land area of 496 square miles, a population in excess of 400,000, and has been identified as one of the fastest growing population areas in the country. It is a suburb of the national capital, a metropolitan area which figures prominently in the normal life routine of most of the people in the area. The entire county, however, cannot be construed as a single community; it is, rather, a broad social context within which people function and which, therefore, provides the place to begin our work.

NATURALLY OCCURRING SOCIAL GROUPS AND INSTITUTIONS. The research of Dr. Howard J. Ehrlich, Sociologist, was directed toward the identification and analysis of the social and political structure in the County. He was particularly interested in the role of voluntary associations in the social life of the county population, and the ways in which they relate to leadership and decision making in county affairs. In M-MHSC-11 (*Studies in Community Social Structure*) two approaches were taken. In the first phase, questions about individuals visible in various leadership connections (in business, real estate, county government, etc.) were included in a sample survey of the County (M-MHSC-9). These data were compared to citations of county leaders in the metropolitan and county newspapers over a one-year period. It was found more than half the reputed leaders (from the survey) were cited in both local and metropolitan papers. This group of reputed leaders received 92% of the total number of citations in the newspapers and 72% of the votes in the survey. There were 18% of those reported in the survey not found in any of the newspapers, and 26% cited only in the county newspapers. From this, three types of leaders were suggested: localites, countians and metropolitans. Localities or countians were more likely to be businessmen. Metropoli-

tans were more likely to be political leaders and to have more newspaper visibility. These data were then to be compared to the results of phase two, the study of "Voluntary Associations and Community Leadership". The first step was to take a census of voluntary associations. There appeared to be over 1200 autonomous organizations that satisfied stringent criteria of voluntary membership and activities. The next step involved the design of a survey to sample the leadership of the associations with regard to their social and ideological characteristics, and to determine the function of the organizations. It was planned that these inquiries would provide a means for a comprehensive study of the nature of voluntary associations generally and would serve as the basis for the study of the role of such organizations in the provision of health and welfare services to the county population. With a delay in the official clearance of the project and Dr. Ehrlich's joining the Department of Sociology and Anthropology at the University of Iowa, further research in this area has been delayed. The work of the Section in the delineation of the social structure, while just beginning, will continue to be an important focus since it will provide particular information on the ways in which services are provided for mental and behavioral disorders. Dr. Goldsmith has assumed responsibility for Project 11.

SOCIAL AND PSYCHOLOGICAL CORRELATES OF RESIDENCE IN METROPOLITAN SUBAREAS. Study of the county is also being approached from the perspective of human ecology. The basic goal here is to ascertain if there are important social and psychological characteristics which can be directly attributed to, associated with or conditioned by residence in specific parts of metropolitan or urban areas. Dr. Harold F. Goldsmith brings the dual skills of a sociologist and a demographer to this area of inquiry. In Project M-MHSC-32 (*Impact of Residence Upon Social Personality Organization*), Dr. Goldsmith is concerned with the delineation of homogeneous residential areas in Prince George's County and the identification of the effects that these areas have on their residents. A preliminary set of social areas has already been identified using Shevky-Bell and

related techniques. Data from the 1962 and 1963 NORC Surveys in the County will be used to evaluate the consequences of residence in the subareas of the County. As a result of this and related analyses it is anticipated that models for projecting the need and demand for mental health facilities can be developed.

Delineation of social areas within the County holds considerable potential for the research program of the Study Center. When these areas have been identified, they can be used in the epidemiological analysis of mental disorder, cases of running away from home and other similar characteristics.

To determine the extent to which the conditions existing in the study population can be generalized to those in other parts of the United States, Dr. Goldsmith is comparing Prince George's County with the Northeastern United States and with other rapid growth counties. This work is being done in Project M-MHSC-33 (*Demographic Structure and the Social Organization of Prince George's County and the Northeastern United States*). Several reports describing the growth, composition and distribution of the population of Prince George's County in relation to the Northeastern United States have already been completed.

For the future, Dr. Goldsmith is planning to study the social and psychological consequences of mobility within and between types of residential areas. The fact that the Washington area has both a high growth rate and a high turnover in housing, makes it an excellent site to explore the inter-relationships of migration, social mobility and individual adjustment. Dr. Goldsmith expects to conduct a large scale survey of mobility patterns of county residents during the FY 1968.

SOCIAL INSTITUTIONS SPECIFICALLY DEALING WITH THE DEVIANT. The study of the development of problems and their management in the community involves a perspective which is situational in character. It requires an orientation to what is observed as behavior, who observes it, how it is defined, and what steps are taken to manage or deal with it.

The most comprehensive study of this sort is that being carried out by Dr. Ann C. Maney. Dr. Maney brings the skills of a sociologist in-

terested in the study of organizations and occupations to evaluation of a therapeutic project in an institution for the mentally retarded, which was searching for programs through which rehabilitation goals might be implemented (M-MHSC-36; *Socialization of Retardates: A Case of Institutional Goal Reorientation*). The first step in the analysis was inquiry into how residents come to be committed to the institution. The assumption in the past had been that intellectual deficit brought about institutional placement. Consequently responsibility for rehabilitation was invested solely in educators who ran programs based on traditional classroom models. The findings of a factory analytic study of the need for institutionalization in this population indicate that socially deviant behavior was responsible for nearly 60% of the commitments, psychiatric disorders were responsible for another 20%, while intellectual retardation alone accounted for only 20% of the cases. Dimensions structuring requests for admission were specified, and target populations for program development were delineated. An instrument for use with other institutional populations is now available. The second step in the analysis was evaluation of the extent to which the on-going socialization process in the Training School modifies those areas of social behavior that were of concern in the community. This has led to development of an empirical typology of dimensions of institutional adjustment and delineation of types of adjustment to be found among residents, which should be ready for publication early in FY 1966. Subsequent analyses will focus on three areas: First, descriptions of the systems of expectations and sanctions confronting the resident and the modifications in these that were deliberately implemented in the course of development of the experimental program in "resocialization"; secondly, evaluation of the impact of the resocialization program on the behavior of residents, and the efficacy of preadmission problem behaviors and pre-experimental adjustment patterns in explaining differential responses of residents to the treatment program; and thirdly, description of the impact of the resocialization program on role definitions of staff, and the ensuing reallocation of functions and au-

thority within the institution. These analyses are planned for the summer and fall, with the goal of a final report in monograph form late in FY 1966.

Dr. Maney has extended her interest in processes of redefinition of function and authority in occupations orienting to new goals in two projects designed to provide a background of findings and hypotheses for future research, one with public health nurses (M-MHSC-37; *An Exploration of Mental Health Roles of Public Health Nurses: A Study of Occupational Contingencies Affecting Mental Health Programming*), the other with pediatricians (M-MHSC-39; *Patterns of Physician Use for Children in a Suburban Population*). The public health nurse study has been carried out in collaboration with Miss Dorothy Boone, Mental Health Nurse from the Clinical Study Section. It is an investigation of the impact of a mental health training experience for staff nurses on the county health department. The pediatrician study is based on a county-wide survey (by NORC) of parents' reports of their use of various kinds of medical services for their children and their reports of the extent to which emotional and behavioral problems were explicitly involved in these services. Reports on the pilot phases of both projects are scheduled for completion in FY 1966. These studies will provide a view of the treatment and management of the deviant through social institutions and occupations whose major orientation is toward their care but who are experiencing changes in the goals of the care they provide.

Perceptions of mental disorder among community caretakers has been of interest since the studies of Shirley Star in the early 1950's. The use of descriptions reported in natural language were used to get at differential modes of viewing cases of mental disorder in a study carried out by Dr. J. R. Newbrough (*Semantic Analysis of the Mental Health Survey M-MHSC-3*). Reports were obtained from ministers, various school officials, physicians, mental health agency staffs, and family service agency staffs in a suburb of Boston about the cases of mental disorder they had dealt with for a calendar month. These reports were evaluated by a language analysis procedure involving measures of abstraction, references to the

life-space setting of the disordered behavior, time orientation and specific occupational terminology. Significant differences were found between job sites and between professional training, indicating that reporters define and describe problems of mental disorder as a function both of where they work and of their training. These findings have led to the development of Project M-MHSC-30 (*Perception of Behavioral Deviance*) where it is the view that reports of mental disorder represent judgments about deviating situations discrepant from the reporter's standards or norms for acceptable behavior. In order to understand why various diagnoses or labels are applied, then, one must measure the standards (values) of the observer and his limits of tolerance around them. Once this is determined, one can then inquire into the ways in which various labels determine the treatment and outcome of disorders. Dr. Newbrough has developed this project in collaboration with Dr. John A. Baldwin and Mr. Geoffrey A. Sharp at the Department of Mental Health, University of Aberdeen Medical School, Scotland. He spent April and May of this year designing a study of the development of perceptions of deviance within the nuclear family and the effects of labelling upon retention or extrusion of the deviant. As background for the study design, Dr. Dee Lloyd with Mr. Thomas Karst from the Ohio State University began a review of the literature on the development of socially-shared concepts and the structural characteristics of language that reflect the level of development of concepts. This will be completed in early FY 1966, and will provide for the appropriate selection of methods for the study of verbal labels as concepts and for the measurement of the repertory of labels available to each observer.

These inquiries provide information on the ways in which problems are perceived and dealt with differently from various positions within the social structure. They seem to indicate that specific occupations have different functions and deal with different problems.

Individual and Group Behavior

In this area we are interested in studying the potential for adequate functioning of indi-

viduals as well as the failures of coping mechanisms. The research is oriented toward inquiries about characteristics of individuals in the population at large, such as achievement levels or anxiety levels, that may be related to behavioral and psychological problems, as well as specific studies of the mentally ill or the deviant.

STUDIES OF PERSONALITY AND SOCIAL CHARACTERISTICS IN THE NORMAL POPULATION. *The Reading Ability and Outcome Study* (M-MHSC-1) began some ten years ago but became inactive. It was revived by Dr. J. R. Newbrough in 1961, when a concerted effort was begun to obtain complete date, to code and punch the data into a form for machine processing, and to design various data analysis procedures. Dr. Dee N. Lloyd, a counseling psychologist, joined the study as Project Associate in May 1964 and became director of the study in January 1965 when Dr. Newbrough was appointed Chief of the Section. It is a population study of 4075 students followed from the 6th grade through their school experience to graduation from high school. The primary purpose is to provide information on the relationships between reading achievement in the 6th grade and later outcomes such as drop-out, lower achievement in secondary school, graduation, and attendance at college. Additional interest is directed toward the effects of age, sex, race, social class and attendance upon achievement and outcome at particular types of schools. Data preparation has been nearly completed; statistical tabulations and analyses have begun. Dr. Newbrough instituted a liaison committee composed of six school system representatives and the project staff. Over a several year period, this committee has served a useful communication function in ensuring that all the source data were obtained, in maintaining sanction, and in serving as medium for reporting results from the study back to the school staff. One of our staff members, Miss Eleanor T. Fay, has produced a report on the development of public education in the county. It is entitled *A History of Educational Progress in Prince George's County, Maryland From Early Times to the Present* and particularly focuses on the educational system's development over

the past 10 years. This was done to provide the proper contextual perspective for the time period in which the subjects were in school (1949-61). A sub-study has been carried out by Mr. Steven R. Tulkin, a research assistant, investigating the relationships of social class, race, and family-related variables to performance on tests of achievement and intellectual functioning. In addition, the differences among various measures of achievement and intellectual functioning are investigated. This aspect of the study is of particular interest to the Prince George's County Board of Education which has collaborated with us on this study by administering the Raven's Progressive Matrices Tests to a large sample of fourth and fifth graders for the purpose of developing test norms. Some of these data will be used to investigate relationships among spatial reasoning, intelligence, and achievement scores, with race and social class level controlled.

The other major study in this area also focuses on the functioning of individuals in school settings, but here the individual difference variables investigated are personality rather than ability variables. The study of *Response Set, Test Structure, and Demographic Correlates of Test Anxiety and Defensiveness in an Elementary School Population* (M-MHSC-27), is directed by Dr. Sheila Feld, a social psychologist. It is the first new project begun in the Section in her program of research directed toward investigating the development and expression of positive and negative achievement strivings in children and the context of the social environment in the home and the school that influence the development and expression of these motivational dispositions. The project utilized a population of 8875 second grade students from the county public schools. It was designed specifically to: provide the necessary baseline data for future research requiring certain measured levels on the Test Anxiety Scale for Children and Defensiveness Scale for Children; investigate methodological issues in personality assessment, specifically acquiescent response tendencies; consider the factor structure of the scales themselves and to develop factorially derived subscales; and investigate the family history and school achievement correlates of the scales in a diversified

population. Data were collected in FY 1964; data preparation and analyses have been the object of work this year. It is planned that reports on this project will be available by mid-FY 1966.

The Reading and Test Anxiety population studies, beyond their specific subject matter orientation, allow for the analysis of information by specific schools, or groups of schools, and the characterization of the personal and social characteristics of the schools. In order to provide basic information for such an approach, we, in collaboration with the Adolescent Process Section and the County Board of Education, instituted *The School Information Project* (M-MHSC-26). This project was directed to the collection of descriptive statistics about each school, its staff and the children for school year 1963-64. It utilized information regularly collected by the County Board of Education, and was carried out during the summer of 1964 by a pupil personnel worker from the Board of Education. With the increasing pool of information available from our research projects, we plan to begin to consider schools as social systems serving particular populations and producing students with specific patterns of achievement, occupational choice, etc. A typology of schools is being considered much in the way PROJECT TALENT views schools as social systems which affect the occupational choices of talented students. This use of schools as important units of analysis illustrates how data can be attacked from both sociological and psychological perspectives.

Dr. Feld also is interested in other substantive approaches to the investigation of the relationships between the motivational dispositions of individuals and the adequacy with which they perform various roles. Project M-MHSC-15 (*Motives and Roles: A Nationwide Interview Study*) was begun at the Survey Research Center, the University of Michigan, in collaboration with Dr. Joseph Veroff and Dr. Gerald Gurin. It is concerned with the relationship between personality structure as reflected in achievement, affiliation, and power motivation and the perceptions of gratifications and problems in marital, parental, and job roles for a cross-section of American adults. In the study *Feelings of Adjustment*

Among Working and Non-Working Mothers (M-MHSC-28), Dr. Feld is investigating the relationship between the work and maternal roles and self-concept. Data are from the sample of county residents interviewed in the NORC survey (M-MHSC-9). She is particularly interested whether these data will substantiate earlier findings from the Survey Research Center that the areas of maternal inadequacy feelings, marital unhappiness, and physical ill-health were the only areas of self-conceptions to differentiate working from non-working mothers, and whether consideration of motivations for working and not working aid in understanding these results. These studies provide a view to the ways in which feelings and attitudes of people in the normal population relate to the ways in which they judge themselves to be adequate; one further step in such research is to obtain independent data on the functional adequacy of these people. Another approach is to study how one's own views and attitudes effect how one actually copes with personal problems. This is being done by Dr. Feld in a study designed to replicate and extend some of the findings reported in *Americans View Their Mental Health*. In project M-MHSC-39 (*Attitudes Toward Mental Health Resources and the Mentally Ill*) the process of self-referral to professional resources for help with personal problems is under study. Questions asked on the NORC survey (M-MHSC-9) were directed toward: estimates of readiness for self-referral, the path of selection of resources, and the attitudes of county residents toward psychiatric resources; relationship of these attitudes to demographic variables and self-evaluations of adjustment; views about mentally ill patients and psychiatric out-patients, and; the relationship of these views to demographic variables, contact with the mentally ill and attitudes toward the personal use of professional resources. In the preliminary analyses directed toward the first two issues, the findings from the Joint Commission Study generally were replicated (e.g. the order of popularity of resources were clergyman, non-psychiatric physician and mental health professional). Reports are expected to be available early in FY 1966.

The studies of individuals in the normal population and groups at large provide baseline descriptive information on the ways in which people typically do things, and the ways in which various personality and social characteristics seem to relate to each other. The identification of such correlates of adjustment and disorder provide then the basis for knowing where to focus intensive studies on various processes of coping.

PERSONALITY AND SOCIAL CHARACTERISTICS OF DEVIANT INDIVIDUALS AND GROUPS. Mental health research has usually started from the clinical case. This has usually focused on intrapsychic processes entirely, because of the clinical background of the investigators. In the Section, with the ecological and social-psychological orientation, we tend to view mental disorder as the result of the complex interplay between personal and social characteristics. In contrast with the population studies, the studies described here focus on individuals who have not coped adequately with the stresses of life.

Dr. Howard J. Ehrlich in project M-MHSC-12 (*Correlates of Dogmatism and Flexibility in a Psychiatric Population*) was interested in the personality characteristics of dogmatism and flexibility in psychiatric patients, the relationship between them and the same characteristics in their therapists, and how this related to the outcome of therapy and hospitalization. Dr. Ehrlich collected the data at the Columbus Psychiatric Institute and Hospital. Generally it was found that patient dogmatism scores were stable through hospitalization and significantly associated with the patient's diagnosis, impairment, treatment, and outcome independent of the patient's socio-economic characteristics. In contrast, the dogmatism scores of therapists appeared to be of no consequence with respect to their behavior towards their patients. Four reports have been drafted.

The other patient oriented work in the Section was begun by Dr. James G. Kelly, former Section Chief. Dr. Kelly was particularly interested in the residents of Prince George's County who sought psychiatric treatment, and in the correlates with their particular station in life. The study of patients coming to the at-

tention of psychiatric resources within the framework of social areas was the basis for developing project M-MHSC-23 (*Community Structure and Patient Status*). The beginning focus of this project was the evaluation of the impact of the opening of a psychiatric ward in the county general hospital. The second phase was to have been the establishment of a continuing analysis of county patient data within the social area framework so that the social and environmental correlates of the development and expression of mental disorder could be established. On the first phase, extensive information on the residents of Prince George's County who sought treatment from psychiatric resources during FY 1961 was gathered from the Maryland State Psychiatric Case Register. This was prepared in tabular form and assembled as a descriptive report on the general characteristics of the county residents who became patients. An amplification of this work was carried out by the Register staff to extend the coverage from FY 1959 to FY 1963. Current plans are to further extend the coverage of this descriptive data through FY 1964, and then to inquire into the experience of cohorts of patients subsequent to their admission (e.g., duration of stay, subsequent releases and admissions, other facilities used after first admission, etc.). Dr. Newbrough is collaborating with Mr. Ben Locke and Dr. Anita Bahn from the Office of Biometry NIMH and Mr. Kurt Gorwitz, Maryland State Register, in the preparation of a report on the use of the psychiatric ward in the general hospital by county residents. Continuing work in this area is planned as a Study Center activity necessitating recruitment of a suitable staff person.

The patient data and case register research function in a similar fashion to studies of normal populations in that they identify the nature and extent of various phenomena, in this instance psychiatric status and type of treatment. This research also provides the basis both for future inquiries into where patients come from (residentially) and whether the proper treatment facilities are available. The latter use is an administrative one which is of particular interest to the county planners for mental health services.

Other Activities

BIBLIOGRAPHIC AND LITERATURE RESEARCH. Dr. Howard J. Ehrlich was particularly interested in the area of intergroup relations. In project M-MHSC-25 (*Studies in Intergroup Relations*) he completed two studies of the instrument errors in the most frequently used question formats in prejudice research. These studies clearly demonstrated that particular item pools and question formats generated a high degree of bias sufficient to alter radically the acceptability of a number of "confirmed" propositions. Dr. Ehrlich collected a bibliography of some 6000 items in the area of Intergroup Relations Research, drafted a critique and conceptual analysis of the concept of "minority group", and began a critical review of the literature on interreligious and interracial marriages. This work is being carried on by Dr. Ehrlich at the University of Iowa.

One of Dr. Ehrlich's previous studies had been devoted to the analysis of the socialization of the police recruit as viewed in the context of role theory. This was M-MHSC-10 (*An Examination of Role Theory: The Case of the State Police*). The major study was reported in book form to be published by the University of Nebraska Press in early 1966. Part of the project included the compilation of an extensive bibliography of literature dealing with role theory. In M-MHSC-55 (*A Bibliography on Role Behavior and Role Theory and Related Materials*) the bibliography is being prepared for publication through the National Clearinghouse on Mental Health Information. Miss Fay and Dr. Newbrough in collaboration with Dr. Ehrlich are preparing an index. It is expected that the final draft will be completed by the middle of FY 1966 and submitted as an official Institute publication.

Bibliographic work in community mental health has been an interest in the Section from the beginning. Before coming to the Study Center, Dr. Kelly and Dr. Newbrough worked with Dr. John A. Baldwin (University of Aberdeen, Scotland) in the compilation of a reference guide to community mental health. In early 1962, Dr. Newbrough and Dr. Baldwin instituted M-MHSC-31 (*A Comprehensive Indexing and Retrieval System for American and European Literature in Mental Health*). This

was to achieve a way of indexing the current literature in research and theory areas relevant to community mental health and to provide a way of searching the indexed collections of the Study Center and the Department of Mental Health in Aberdeen simultaneously. A system based on the use of a vocabulary of descriptive terms and a coordinate indexing approach was designed and the vocabulary generated. In 1964 the National Clearinghouse on Mental Health Information became interested in the project as a major source of input for its searching, retrieval and bibliography generating system. Tests of the indexing and retrieval efficiency of the present system will be carried out this summer and an operational system is planned for late FY 1966. This effort holds the potential for efficient and exhaustive searches of literature relevant both to our research needs and to the more general needs of the Institute.

PROFESSIONAL ACTIVITY. In the area of professional activity, Dr. Newbrough has been active on the committee planning the Conference Preparation of Clinical Psychologists to be held in August, 1965. He has been the official representative of the Corresponding Committee of Fifty, Division of Clinical Psychology, American Psychological Association, and was appointed to be in charge of recording the proceedings of this national conference. During the year, Dr. Lloyd and Dr. Newbrough were asked to write a review of the national conferences held on graduate training in clinical psychology. This review was published as the lead chapter in the pre-conference report published in April 1965.

Dr. Harold F. Goldsmith is currently on the editorial review board of the journal *Rural Sociology*. He also has been participating in the development of the research program of Northeast Regional Committee 47. This committee is sponsored by the Agricultural Experiment Stations of ten land grant universities in the Northeast and by the U.S.D.A. It is concerned with the social and economic consequences of changes in employment upon selected Northeastern communities.

The work of the present and former Section Chiefs include attempts at conceptualizing the

field of community mental health and the purpose for services. Thus, Dr. Newbrough organized a symposium on the general area of community mental health for the IXth Inter-American Congress of Psychology held in Miami, December 1964. He prepared the first paper entitled *Community Mental Health: A Movement in Search of a Theory*. The other three papers covered practice, research and training. Discussants were two Latin Americans: Dr. Rene Gonzales, WHO Regional Consultant in Mental Health and Dr. Mauricio Knobel, Professor of Psychology, National University of La Plata, Argentina. Dr. James G. Kelly's paper prepared for the Tenth Symposium sponsored by the Group for the Advancement of Psychiatry entitled, *The Mental Health Agent in the Urban Community* has recently been published as a GAP report.

Adolescent Process Section

The existence of the Adolescent Process Section is predicated on the premise that in mental health our data about adolescents come almost exclusively from our knowledge about the casualties to the school system or those whose troublesomeness in the community creates problems of national concern. There is a need to broaden our perspective on adolescence to include those who have not as yet or who may never come to the attention of the authorities. The selection of "Adolescent Process" for the title of the section is recognition that adolescence, especially in the social and psychological definitions of that term, is a continuing process. Physiologically adolescence is a process also, but the hormone-guided changes for any individual commence at a given time and are pretty well over with by the late teens or early twenties. The social and psychological processes of accommodation from the status of childhood to that of adulthood are more elusive and once set in motion may continue as a dominant influence throughout the adult years. The origins and terminations of these processes are very much determined, on the one hand, by how early the child is pressured toward self-sufficiency, and on the other hand, to the extent that transition into adult occupations and status is prolonged.

Out of this orientation several projects have emerged. Each required a unique solution to the problem of establishing and maintaining continuing research in a host community. In doing so we have tried to meet an often ignored responsibility of social and behavioral scientists in putting their work to the test, that is developing means by which to increase its social utility. This may involve persuading powerful figures to delay judgment and action until data systematically and carefully collected can be brought in, at the same time guarding against the temptation to seek methodological perfection and theoretical completeness. It is this intellectual endeavor, so well trained into the social scientist, that often leaves the men of social action and social responsibility, if not skeptical, cynical about the potential contributions of their scientific advisors. It is not easy to do research under the pressure of deadlines for decision, nor can one blame the social scientists who fall prey to their own reverberating perfectionism in the face of the enormity of social problems and social responsibility.

Our study of runaway children or more precisely children reported missing from home illustrates one way the method of science can become part of the values and thinking of policymakers.

Several years ago the Circuit Court Judge handling juvenile matters voiced alarm about the increasing number of runaway girls that had come to his attention, some fifty in the previous year. Sharp interest was shown in why children run away from home. It was concluded that speculation could go on *ad infinitum* but the answers awaited a systematic examination of the phenomenon. The idea of a research project caught the Judge's interest and within a month the Police and Probation Departments were for it as well. A group, consisting of representatives from the judiciary, probation, police, the school system and the Adolescent Process Section met to plan the study. Everyone had his idea as to why children run away. Some said there were more boys than girls; others said there were more girls than boys. A consensus developed that they came from poor homes, were school dropouts, police problems, and that for all intents and purposes it was the same group that was

in trouble elsewhere in the community. It is remarkable how rapidly the "hard core" concept is invoked to describe any deviance in our society.

Subsequently the group met twice more to refine its speculations into rough hypotheses about why children run away. The meetings were most helpful to the research staff. The police officers were embarrassingly precise in the definition of variables. They, along with the judge and probation people, easily translated the exacting demands of rules of evidence into scientific skepticism. The group process allowed all to rediscover that any act is multiply determined, and that the same act expressed by many individuals inevitably is found to have a multiplicity of explanations. The group was preoccupied with the validity of the data and in this preoccupation found themselves willing to change time-honored bureaucratic procedures which only *they* had the power to change; to change quickly and effectively. For example, up until the beginning of this study the police routinely took missing persons information over the telephone; had no separate method for recording it; and filed reports of *all* complaints whether it be of a missing child, an assault, a car theft, or a murder in consecutive order. For this study they now saw the need for a separate form and files for information on missing persons. They also saw the need to standardize the data-collecting procedure to get consistent and accurate information. In the course of one meeting the police decided on a radical change in their missing persons procedure. Instead of taking information by phone, a uniformed officer in a squad car is now sent to the home of the complainant, usually the parent or spouse of the missing person. All officers are trained in the use of the new data-gathering instrument. The change was effected within a few weeks.

That was in the summer and fall of 1961. Since February, 1962, the police have been sending the Adolescent Process Section duplicates of all forms on missing persons in the County. Follow-up interviews with parents of children reported between July, 1963 and July, 1964 have been completed by our staff (M-MHSC-21). Sanction for the study, com-

ing from the police officers on the beat all the way up to the County Commissioners (who at one time even appropriated \$3,000 during a period of critical shortage), has contributed to the maintenance of high project staff morale and has played no little part in enabling us to obtain a 97% completion rate on our follow-up interviews.

Over the three year period just ended this past February 1, some 2,944 missing persons reports were filed by the Prince George's County police; 38% on adults, 62% on children. Over time the proportion of adults has risen slightly (from 30 to 42%). Although for adults the sexes are reported with almost equal frequency, boys hold a 60-40 lead over girls in the juvenile division. However, the proportion reverses in the young adult age-range. Young women between 18 and 20 as well as between 21 and 29 are reported one and a half times as often as their male age-mates.

One year was selected for intensive study. Called the "sampling year," it extended from July 25, 1963 through July 24, 1964. Sixteen hundred and seventy reports were filed; 990 of them on children. In all 831 individual children produced those reports; 103 children contributing more than one report during that time period. That is, only 12% were repeaters. Over three-quarters of all the children were age 13 to 17, and in junior and senior high school. According to follow-up interviews with parents about 80% had run away from home, thus giving rise to the report of a child missing. About four out of every ten reportedly ran away to "avoid or postpone specific punishment or responsibility for past behavior or because of anger and resentment over treatment at home." Putting it another way—reasons given for leaving fall almost equally into the categories of *avoidance of responsibility, limits and discipline (28%), reactions to limits and discipline (23%), and embarkation on an excursion or project (28%)*, usually not sanctioned by parents. In all, the view we get, through parent-filtered perceptions, confirms prevalent notions that running away is a strategy employed in intergenerational conflict.

Information on how frequently it is used in the adolescent population at large awaits the collection of questionnaire data from a sample

of junior and senior high school students; how seriously running away should be viewed, depends on the analysis of correlative information from schools, court and police sources. Before a representative sample for the questionnaire phase could be selected, data on the characteristics of all county secondary school populations had to be obtained. In a project entitled School Information Project (M-MHSC-26), the Adolescent Process Section joined with the Community Projects Section to collect such information. Completed in February, 1965, the data on students and schools serves as baseline for school attendance and performance information collected on runaways.

We have found that it is more useful to think of "adolescences" rather than a single adolescence; that the process of making the transition or accommodation from childhood to adulthood is going to vary, depending upon the point of origin and the point of arrival, that this is not uniform across social classes or between sexes. Therefore it makes more sense to talk about various adolescences and to try to keep this distinction in mind while making generalizations from the data of a particular study to an entire segment of society. One of the on-going activities of the section has been to review the literature on adolescence and to develop a format for researches which would utilize representatives of various "adolescent processes" existing in a given community. Dr. Joan Snyder, an anthropologist formerly our staff fellow, produced a design for such a study (M-MHSC-35). It focuses on the concept of "the future"—how important it becomes and how it affects the choice of friends and activities as well as the personal adjustment of a child. The design is so constructed as to include both girls and boys from three operationally defined social classes and five age levels ranging from ten to eighteen. Life style interviews would produce data on the shifting influences, allegiances and conflicts in the several spheres of an adolescent's life; his home, his school, his friends, his job. Whether this most interesting project is carried to fruition depends upon future availability of resources.

Another study (M-MHSC-22) focuses on one of the prime influences among adolescents,

the peer group. A current vogue is to devise "community" programs for adolescents, or for any other group set aside because of its Troublesomeness to the rest of society. But "community" is something of a vague concept, which often boils down to that class of persons (adults) who are most visible, most accessible to outsiders, or most conspicuously respectable, and who purport to be interested in changing the lives or behavior of the appropriate class of other persons in a given, often vaguely defined, geographic area or geo-political jurisdiction. Financial resources by the millions and personnel by the thousands are fed into these "community" machines for effecting behavior change. The outcomes are often unconvincing.

We thought we might look at community from the other way around; namely, go to the adolescents and evise some method for getting them to define what *their* community was. The concept of "effective community" is being used to distinguish this personal community from the adult-defined community most visible from the outside. This project has been under the leadership of Derek V. Roemer. It has led us into an experiment with data collecting methods whose import as a vehicle for changing the behavior of adolescents may very well equal their usefulness in the scientific process. The study population consists of all boys ages thirteen through eighteen living in an all-Negro, predominantly lower-class network of neighborhoods adjacent to Washington, D. C. The boundary of the area is fairly clearly defined by the terrain on one hand and racial barriers to interaction on the other. The object has been to get all of the boys to tell us how they spend their time and with whom; and on an empirical basis identify the "settings" used by this population for their informal non-adult-dominated activities. We are interested in the hangouts, the basketball courts, the football fields, the corners and back alleys where dice are thrown, where the older and younger hang out to talk about life, the future, what one can expect, how to make it, why one didn't. Once identified, the settings themselves are to be studied. The loose groupings of boys who use a setting or system of settings will be identified, and the priorities of interest and values associated with them examined.

The approach made to this lower-class Negro group is one not often employed by middle-class whites. We went to them to *get* help, not to offer it. We told them what we wanted, we set tasks that they could do, we trained them, and paid them for doing it. Currently we have about three hundred boys on "our payroll". So far we have disbursed several thousand dollars in collecting data on the comings and goings of this population. The natural, respected or feared leaders were identified and they in turn became team leaders responsible for data collection from ten to twenty boys each. Rewards, such as staff parties, dances, etc., have served to reinforce the fact that we valued the "legitimate" work these boys have been doing in the service of science. The result has been that Mr. Roemer has developed a remarkable relationship with most of the boys, based on mutual trust. They have a comfortable knowledge of what his motivation is in working with them. They feel he accepts them as they are, and is not patiently or impatiently awaiting their conversion to a middle-class world.

Data take the form of "itineraries" collected daily for seven-day time periods at different seasons of the year. In addition to descriptive analysis of behavior in various settings and of the psychological habitats of various types of individuals, a factor-analytic technique is being adapted to identify *patterns* of grouping, both in terms of persons and the places in which they behave. Such patterns will in effect define different "effective communities" within a single social area. That information will permit study of the status criteria which are hypothesized as partially determining associations among the study population.

The project's current theoretical rationale sees the peer group as a major influence determining the specific composition of the set of persons and places making up a given individual's personal community. Thus the factors determining the individual's location in a particular peer group become important explanatory variables. A theory of peer status criteria is being developed to incorporate these ideas, and new instruments are being designed to supplement the itineraries in the appropriate substantive areas.

Following up our interests in lower-class Negro adolescents, we have provided continuing support for a project dealing with lower-class Negro adult males in the city center. Elliot Liebow, an anthropologist, has spent over eighteen months on the street corner with a number of lowest income Negro men. This study (M-MHSC-59) has two general objectives:

1. To identify the life styles, and the values and attitudes which lie behind these life styles, of the urban, low-income, street corner Negro.
2. To determine whether these life styles and value systems are primarily located in a self-generating, self-supporting, and relatively independent cultural and social (Negro) sub-system, or whether they are located directly in the larger, American, middle-class dominated society.

The data were collected through eighteen months of participant observation in a four-block area of Washington, D.C. The great bulk of the material is drawn from some two dozen adult Negro males who shared a corner in the Second Precinct as their base of operations. These men were unskilled construction workers, casual day laborers, menial workers in the service trades, or unemployed. They include single and married men, some of them living with women and children, some not.

The main body of data constitutes a record of the day by day routines of these men—what they said and what they did—on the street corner, the alleys, the hallways, pool rooms, beer joints, and in private rooms and apartments. These data are descriptive, narrative, and anecdotal. They span the four seasons of the year and all hours of the day and night. All observations were dictated on tape away from the field situation (but with the knowledge of those being observed) and subsequently transcribed.

In addition to the main body of materials, some 40 hours of taped informal interviews were collected. Life history materials make up an important part of these recordings.

One gross fact that is emerging from the on-going analysis is that streetcorner values are

strikingly similar to the dominant middle-class values of society at large. The evidence points to the fact that the streetcorner Negro is not simply a carrier of an independent cultural tradition but rather that his distinctive life styles and behavior patterns are in large part the direct response to the world he lives in of one who is black, poor, and unskilled.

The determination to eliminate delinquency and dependency from our national life has fostered a growing body of literature on delinquency and family life among the urban poor. But this literature deals only incidentally with the adult male. Delinquency research normally focuses on the pre-adult; studies of family life among low-income urban poor tend to deal with "female-centered" households and almost invariably depict the low-income urban world as one peopled mainly by women and children. The fact that the adult male is a living, available model for adolescents to emulate or deplore has not received sufficient recognition.

The men described here represent one of several possible models for low-income Negro adolescents. Though he may be a figure who drifts in and out of the life of the family members, he has a significant influence on his domain, the streetcorner. In making the adult male the direct subject of investigation this study should help provide balance and representativeness to the available literature.

By relying on participation and direct observation this study avoids the problems and distortions inherent in attempting to draw members of the lower class into "the net of idle-class participation" that interviews and questionnaires necessarily require. The determination of lower-class adult Negro life styles and values, and their origins is crucial to any purposeful attempt at controlled intervention.

The social reality about runaway children looks a bit different than it did at the outset; they aren't all girls or hard core delinquents. Similar preconceptions about lower-class Negro youth and men are being put to the test as well. The ultimate discovery may be that the populations and phenomena we have chosen to study are complex ones; that stereotypical thinking, whether it is used to describe runaway children, lower-class Negroes, or adolescents generally, is going to be unproductive

and socially useless from the point of view of the responsible and humanitarian public administrator. Though adolescents, runaways, or lower-class men may be conceptually or statistically sub-divided into groups with like characteristics it is unlikely that this exercise will allow for the routine assignment of dispositions easily automated. Rather, our researches are likely to reaffirm the need to consider each adolescent as an individual living in unique circumstances. Much of the burden for actually effecting behavior changes still rests upon the ability of judges, school authorities, police, health and welfare agents to act wisely in each individual instance. In the final analysis, no research can substitute for *this* type of wisdom.

Two unexpected but nevertheless gratifying results, arising directly out of the missing children study, have been the increased importance placed on research by the court and the use of our staff in training police. The judge introduced a bill in the state legislature authorizing Circuit Court Judges to order County Commissioners to engage in research on social problems. Though this authority has not as yet been exercised it nevertheless stands as a testimony to both the judge's and the legislators' commitment to social science research. Similar data gathering approaches dealing with adoptive children and divorces are planned. An attempt to evaluate Juvenile Court procedures by means of a careful follow-up study was initiated recently. At the police department's request we assisted in setting up a training program for forty-five officers designated as a civil disturbance unit. The men were being prepared to meet repercussions from the civil rights movement and the demonstrations already occurring in the County. Though this project is formally terminated, section staff still maintains active liaison with the police and monitors their handling of incidents relevant to their training (M-MHSC-58). Reports of the project will appear as a chapter in a book of readings entitled *Minority Problems* (ed. Arnold Rose), and in the *Journal of Applied Behavioral Sciences*.

Other activities of the Adolescent Process Section include continued participation on the Interdepartmental Committee on Children and Youth, Sub-Committee on Services for Adoles-

cent Girls; and the role of advisor to the National Health Survey's upcoming study of adolescents. In June, 1964, the section planned the workshop program for the 27th Annual Conference of the National Council of Juvenile Court Judges. Five of the six panels brought to the 300 judges the results of social and behavioral science research on delinquency, judicial dispositions, and adolescence. Outstanding so-

cial and behavioral scientists were recruited and brought to the Conference by the section.

The section, then, has embarked upon an excursion with dual purposes: to increase our knowledge of adolescence through systematic and innovative observation, is one. To conduct our investigations in such a way as to involve the policy makers rather than invoke their scorn and fear, is the other.

NATIONAL INSTITUTE OF NEUROLOGICAL DISEASES AND BLINDNESS

INTRODUCTION

The Intramural Research activity of NINDB has continued to operate on a relatively stable base during this 12-month period. Comparisons with the previous Annual Report should take into account the 18-month base of that report due to the shift from calendar to fiscal year. The 10 Laboratories and Branches of the Intramural Program in Bethesda continue to carry 242 positions. One hundred and seven of these are professionals at the level of GS-11 or higher, or equivalent. Using this level to divide professional from nonprofessional employees we have an average of 1 1/4 nonprofessionals per GS-11 or higher employee. Space available to the program has increased by about 900 square feet or approximately 3 percent. The Intramural Budget taken on a 12-month base has increased by 2.8 percent.

In some areas of the program there are many well-qualified young recruits available, in others recruiting appears to be more difficult. The competitive position of NIH with regard to recruiting varies in the different specialties. One of the most important measures of success of the program is its ability to attract good people and this appears to be as good as ever.

The present Acting Associate Director feels that it is important to the program that a permanent Scientific Director be found and has asked to be relieved of his present duties by January 1, 1966. A number of candidates have been suggested for this position and two have been interviewed.

Twenty new people have been employed during the year to replace those who have left. Thirteen of these are clinical or research associates or staff fellows. The remaining seven have been given Civil Service status. Thus, there are now seven fewer temporary positions available to the program; without further expansion there will be a smaller turnover of personnel next year. One result of these forces is

the increasing average age of the Intramural Staff. Of the 107 GS-11 or higher employees the average age last year was 36.3 years, while the average for the current reporting period is 37.2. This trend will presumably continue until new space and position are made available or until a plateau is reached of very mature individuals. In order to combat this trend an effort should be made to replace permanent employees who leave with temporarily filled positions wherever possible.

Some additional space may become available when the new Clinical Center Library quarters are occupied, probably early in 1967. Completion of the new NIMH/NINDB Building 36, scheduled for early 1968, will provide a large increase in space for the Intramural research program. The very great potential represented by this space increase must not be wasted by assigning it too quickly to existing Laboratories and Branches. Slow and steady expansion will have the maximum vitalizing effect on this research organization. There is an eternal conflict between the need to make the best use of available space and the need to keep it from slipping away.

The number of research projects reported this year is 192 in comparison to the 170 reported last year despite the 50 percent longer reporting period of the previous year. While the greatest assets of NIH continue to be the opportunity for collaboration, bringing a number of different specialized disciplines to bear on a single research objective, evidence that the intramural program is availing itself of such opportunities is indicated by the fact that 101 of the research projects currently active involve collaboration with other laboratories and branches within NINDB, within NIH, and with outside organizations. About half of these represent collaboration with other institutes, one-third show collaboration with laboratories outside NIH.

The number of projects reported as completed and/or terminated is 59. This number is difficult to interpret but represents a shift of space and manpower to new projects in the continuing competition between new and old research operations.

The newest addition to the NINDB intramural research program is the Laboratory of Perinatal Physiology in Puerto Rico under Dr. Ronald E. Myers. This laboratory, formerly under Dr. William Windle, has not been included in the above figures, which showed no change in space or positions, because it is being transferred from the collaborative and field program. The laboratory occupies temporary quarters in the U.S. Public Health Service

Quarantine Station in San Juan and operates several islands around Puerto Rico. The laboratory is scheduled to be housed in a new building to be erected in the Centro Medico in close proximity to the new University of Puerto Rico Medical School and other functionally related organizations.

It is difficult to recognize the most important research achievements of the intramural research group, perhaps the best judges of this are the laboratory and branch chiefs themselves. The annual accomplishments and plans for future research are described in summaries preceding the individual reports of each laboratory and branch.

Intramural research NINDB

Program	Persons		Dollars in thousands		Space square feet		Beds	
	Fiscal year 1964	Fiscal year 1965	Fiscal year 1964	Fiscal year 1965	Fiscal year 1964	Fiscal year 1965	Fiscal year 1964	Fiscal year 1965
Office of Associate Director-----	26	26	250	243	2,494	2,920	-----	-----
Miscellaneous-----			264	103				
Subtotal-----	26	26	514	346	2,494	2,920	-----	-----
Med. Neuro.-----	25	25	290	360	12,675	12,641	26	26
Surg. Neuro.-----	51	51	600	605	15,425	15,488	26	26
EEG-----	12	12	107	130	1,697	1,697	-----	-----
Ophthalmology-----	34	34	394	396	13,800	13,800	26	26
Subtotal ² -----	122	122	1,391	1,491	18,597	18,626	78	78
Biophysics-----	13	13	175	212	1,530	2,178	-----	-----
Neuroanatomy-----	30	30	293	348	7,130	6,907	-----	-----
Mol. Biol.-----	15	15	162	194	3,328	3,328	-----	-----
Neurophysiol.-----	11	11	149	153	2,035	2,035	-----	-----
Neuropathology-----	5	5	54	57	1,239	1,211	-----	-----
Neurochemistry-----	20	20	258	264	3,200	3,205	-----	-----
Subtotal ³ -----	94	94	1,091	1,228	18,462	18,864	78	78
TOTAL-----	242	242	2,996	3,065	34,553	35,410	-----	-----
Perinatal Physiology-----	67	67	737	746	20,461	18,289	+109 acre	+109 acre
GRAND TOTAL-----	309	309	3,733	3,811	55,014	53,699	78	78

¹ These figures do not include Clinical Center space (patient wards and surgical wing, Building 10A) occupied by the Branches indicated.

² Clinical program.

³ Basic program.

On April 16, 1965, the Laboratory of Perinatal Physiology was transferred from the Collaborative and Field Research Area to the Intramural Research Area.

MEDICAL NEUROLOGY BRANCH

Clinical Investigation Program

Introduction

The function of this program is to apply the most promising basic research techniques to the clinical problems of the patients. An interrelated multidimensional attack on the target diseases is essential. Added to the techniques of *histochemistry* and *tissue culture* have been *biochemistry* and *immunology*, but in quite modest forms due to limitations of space and personnel. The techniques of *electron-microscopy* and *autoradiography* have been begun only on a collaborative basis, due to acute lack of space for these important investigations. We are very appreciative of the collaboration received in these and other techniques. It is obvious that to have a balanced clinical investigative program, each of these techniques must be provided for more adequately.

For the clinical investigations, 275 patients were admitted for a total of 6,786 patients days, and 744 out-patients were seen. There were 296 muscle biopsies obtained.

The clinical neurologists carried a considerable service responsibility. They provided 356 consultations to other departments, and performed the indicated myelograms, pneumoencephalograms, and cerebral angiograms on those patients.

The 2-year approved residency training program in clinical neurology has continued; medical students and residents from Howard University were taught clinical neurology weekly; and technicians and investigators came as guest workers to learn clinical research techniques in neurology and especially the application of enzyme histochemistry to human neuromuscular disease.

Myopathies

A completely new classification of the myopathies based primarily on metabolic aspects has been developed. Treatment of myopathies associated with collagen-vascular disease has been rather successful in some patients, and in certain cases resulted from reduction of the

medication they were on; appropriate treatment was chosen on the basis of clinical judgment. A symposium on *Current Concepts of the Myopathies* has been guest-edited and seven chapters written by our neurologists; it is also being published as a separate monograph. Chapters summarizing our total experience on "Histochemistry of Neuromuscular Disease" have been written for two texts. An even more comprehensive Atlas summarizing muscle biopsy histochemistry of more than 1,000 patients with neuromuscular disease is being prepared. Many new histochemical findings have been described, compared, and contrasted in a variety of myopathies including hereditary dystrophies, collagen-vascular myopathies, endocrine myopathies, and certain rare myopathies. Studies have been made of selective fiber involvement according to histochemical type in certain myopathies. Basic histochemical mechanisms have been analysed in human (and some animal) skeletal muscle, including direct Nitro-Blue Tetrazolium binding, the phenazine methosulfate shunt, and various ATPase reactions. A method for isozyme histochemistry of lactate dehydrogenase has been devised. Detection of subtle abnormalities with the histochemical reactions has pointed up the great need for more studies of normal human muscle, which we are commencing. Molecular abnormalities of myoglobin and muscle lactate dehydrogenase were found not to be specific for muscular dystrophy, nor was decrease in total body K⁴⁰. Turnover of several serum proteins was studied; excessive catabolism of γ -globulin was found in nearly all patients with myotonic dystrophy and in none of the other neuromuscular disorders studied. Accuracy of detecting asymptomatic carriers of muscular dystrophy was evaluated using serum creatine phosphokinase and muscle biopsy histochemistry studies. Tissue culture was used to study various aspects of skeletal muscle cell biology, both of chick embryo and, to a lesser extent, of human tissue. Electron-microscopy of abnormal human muscle is in the early stages. A technique has been devised for histochemical control of the specimens at several stages of their processing for electron-microscopy.

Episodic Weakness

A new classification of the periodic paralyses and non-dystrophic myotonias has been developed, based on the provocative and therapeutic effects of various ionic unbalancing tests. The tests are designed to determine the identity of non-identity of these various clinical conditions, based on the more accurate metabolic parameters. The therapeutic trials indicated by the test results are being run in these conditions. Forearm studies are planned for the future to study more selectively muscle metabolism in these patients. Muscle biopsies obtained between and during attacks have been investigated histochemically, and electron-microscopy is in progress. Histochemical studies make it doubtful that structural changes in the muscle fibers are responsible for the weakness in the initial part of the attack. The new pathological entity "mitochondrial aggregate" has been described in patients with these conditions, and its specificity is being analysed. In a patient with succinylcholine-induced paralysis, the low serum cholinesterase was found by electro-phoresis to be associated with a newly recognized selective absence of the fastest two cholinesterase bands.

Myasthenia Gravis

A number of immunologic techniques have been set up to study immunologic aspects of myasthenia gravis, as well as of myopathies in collagen-vascular disease. Immunologic techniques are also being used to study protein abnormalities in other neurologic disorders, such as ataxia telangiectasia and the progressive peripheral neuropathy of primary amyloidosis. More emphasis on studies of the altered cellular aspects of immunity in neurologic disease is planned. In myasthenia gravis, with a very careful fluorescent antibody study, the muscle-binding factor in myasthenia gravis was *not* found to be bound to the neuromuscular junctions. Clinical and immunologic aspects of non-myasthenic patients with thymoma are being studied to seek subclinical evidence of myasthenia. Histochemistry of muscle biopsies from 38 myasthenia gravis patients showed that virtually all were abnormal, with denervation atrophy and type II fiber atrophy (a newly de-

scribed histochemical change) being far more common than muscle fiber necrosis or cellular reactions (lymphorrhages).

Amyotrophic Lateral Sclerosis (ALS), and Other Diseases Affecting the Lower Motor Neuron

A monograph is in preparation. The first part is a review of the entire literature on ALS. The second part covers our special investigations, clinical and basic, of ALS. Clinical data on 200 patients are being analysed by an inverted index system using a specially developed vocabulary. In the first 100 patients, 10 percent were found to have neoplasms. In about 150 patients, approximately 25 percent have abnormalities of carbohydrate metabolism, the mechanisms of which are being sought. Intolerance to amino acids and other metabolites is being sought, as are abnormalities in protein, lipid, and cyanocobalamin metabolism. For therapeutic trials, an apparatus giving reproducible quantitation of muscle strength has been developed and double-blind evaluation of several methods of treatment (as well as placebos) is nearly completed. Metabolic parameters of biopsied motor neurons from ALS patients are being studied by autoradiography, histochemistry, and biochemistry; and neuronal ultrastructure is being explored by electron-microscopy. Control neuronal material is obtained from patients with other diseases and from normal apes. The histochemistry of animal motor neurons in tissue culture is being studied. The changes in histochemistry and lactate dehydrogenase isozymes in animal muscle after denervation have been described.

Neuroradiology Section

Radiographic Diagnosis

A project aiming at an appraisal of the vascularization of spinal cord lesions is under way. Angiography with selective catheterization and with subtraction is being carried out for this purpose in patients with arteriovenous malformations and tumors of the spinal cord. The *Atlas of Pathologic Pneumoencephalographic Anatomy* is nearing completion. A cooper-

ative project is being initiated on growth hormone effects in dwarfs. Repeated sella-turcica measurements will be taken in the patients so treated. A reevaluation by computer techniques of the angiographic patterns of superficial cerebral veins in the two hemispheres is under way. The aim of this project is to reevaluate on a very large clinical material the previous finding that the angiographic patterns of superficial discharging veins in the two hemispheres are different, and that a relationship of these morphological differences with the cerebral functional dominance exists. Further experience is being gained with the useful refinement of pneumoencephalography developed in this section, namely "axial transverse encephalography".

Radiation Dosimetry

A system is being evaluated to measure secondary radiation from irradiated residual x-ray opaque material in the spinal canal. Among the solid-state radiation detection devices, the thermoluminescent lithium fluoride (LiF) crystals have been chosen.

Isotopic Diagnosis

A clinical comparison of radio-iodinated serum albumin and technetium ^{99m} pertechnetate as brain scanning agents is under way. The aim of this project is to evaluate the relative merits of two widely used radioactive tracers, RISA and technetium ^{99m} pertechnetate. This appraisal will be as objective as possible in clinical material, due to the fact that the double isotope-double scan technique is being used, i.e., two tracers are injected in the same patient, each injection being followed by a scan. Radioactive isotopes, especially technetium ^{99m} pertechnetate, and brain scanning have been used to evaluate the patency and morphology of cerebrospinal fluid shunts. For the first time, the shunting paths have been actually visualized. Further experience has been accumulated in isotope-ventriculography and isotope-cisternography. These have proven to be diagnostic tools permitting the morphologic and dynamic study of the cerebrospinal fluid pathways more accurately than was ever possible before with any other diagnostic test.

With the adjunction of our new multiprobe detecting device—the "Tetrascanner"—the mapping and timing of the cerebrospinal fluid circulation is being studied in great detail. Stress is placed upon the pathologic dynamics of the same fluid. Virus-induced experimental brain tumors, namely intracranial sarcomas and gliomas, have been produced in dogs. This appears to be an ideal biological system for a comparative evaluation of the many radioactive tracers used or potentially usable in human brain scanning. Such potential tracers will be screened in these animals. The observation that technetium ^{99m} pertechnetate has a very high specificity for salivary glands has brought about a project in which salivary gland scanning is being evaluated. Emphasis will be put upon salivary gland tumors and other salivary pathology. The general plan has been prepared and a few chapters have been written of a monograph dealing with the newest aspects of brain scanning.

Neuropharmacology Section

This section has continued its research program in relation to the physiology and pharmacology of muscle. We have found that at least certain smooth muscles, as well as slow striated muscles, develop contracture in response to calcium deprivation. The response may not be related directly to electrical activity. The findings thus indicate that intracellular calcium movements may control tension development. These studies have necessitated the recording of electrical activity in slow skeletal and smooth muscle; new methods for these measurements have been developed and used. The ionic requirements for relaxation of slow skeletal muscle have also been studied, and much data is not known as yet and they thus remain have been obtained. The meaning of these data descriptive.

A comparative pharmacological study of fast and slow types of twitch muscle has continued to a limited extent. The study involves further instrumentation development. A study of the genetics of serum cholinesterase has been made in a family with serum cholinesterase abnormality.

Mechanical properties of muscle have been studied as a prerequisite to the evaluation of

drug action. Unexpected differences have been found between the elastic components of fast and slow twitch type muscles. The mechanical properties derived from dynamic elastic responses were analysed in terms of physical elastic models. A suitable technique for limb joint fixation in animals has been developed. The method provides a modified mechanical environment for the muscle in the presence of an intact neural apparatus. Immobilization in slower muscles produced atrophic and mechanical changes. These studies are in progress and are to be continued to include the faster responding muscle.

Perfusion of isolated portions of the cerebral ventricles in cats with pharmacologic agents has been further refined. The effects of various agents on cerebral physiology have been recorded, and the pathophysiology of the induced myoclonic jerks investigated. Future plans call for more precise localization of the pharmacologic agents by the use of radioisotopic and fluorescent markers.

SURGICAL NEUROLOGY BRANCH

During the period of this report, the Branch conducted investigations under the following categorical titles; cerebral trauma, developmental defects, epilepsy, involuntary movements, brain tumor, cerebral edema, language and memory, effects of low temperatures, microbial analysis of neurosurgical environments, and neurosurgical monitoring. Twenty-seven reports were prepared for publication.

As of this date, 22 April 1965, 202 persons participated in the clinical investigations as inpatients, and 443 outpatients were examined in a total of 568 visits. There were 110 major operative procedures.

Branch investigations have been conducted in active collaboration with the following organizations: Branch of Electroencephalography, NINDE; Laboratory of Neurochemistry, NINDB; Laboratory of Neurophysiology, NINDB; Biometrics, Branch, NINDB; Clinical Center (Departments of Clinical Pathology and Diagnostic X-ray); Instrument Engineering & Development Branch, DRS, NIH; Computer Facilities, DRS, NIH; Office of Medi-

cal Examiner, City of New York; Walter Reed Army Medical Research Institute; Army Medical Research Laboratory, Ft. Knox; Bureau of Standards; Personnel Protection, David Taylor Model Basin, Department of the Navy; U. S. School of Aviation Medicine, Department of the Navy, Pensacola.

Cerebral Trauma

Investigation of the effects of cerebral trauma is continuing in the laboratory and in the clinic. In the laboratory, the nature of the relation between cerebral concussion and the impulse of the blow is being studied. An experimental model of cerebral concussion in the monkey is now being tested under a variety of conditions in order to better understand the mechanics of concussion. The impact force, velocity, linear acceleration of the head, intracranial pressure changes, and head displacement have been measured. Based on this data, a theoretical model for experimental head injury is being developed.

Continuation of radiological studies after carotid angiography revealed that the marked slowing of circulation described in last year's report (within the period of 15-30 minutes after trauma) is of early onset (within 1-2 minutes of the blow) and appears to parallel the development and persistence of post-traumatic cerebral edema.

Clinical studies suggest that persons recovering from the effects of severe deceleration injury without additional complication, display an accelerated recapitulation of postnatal life history. This seems particularly true where bilateral temporal involvement is a feature of the traumatic lesions.

Developmental Defects

Clinical, biochemical and genetical studies of mental retardation, and progressive cerebral degeneration and cerebral palsy; certain pathological lesions occurring during prenatal, intranatal and early postnatal life; a cytogenetical study of human chromosomes, especially in patients with congenital malformations and mongoloids; cranial measurements in males and females; and a biochemical study of the amino acid analogue, Trifluoroleucine, are sub-

titles under which problems of developmental defects have been approached during the past year.

The investigation of ten patients with Hurler's syndrome revealed that autosomal recessive and sex-linked varieties cannot be distinguished by the type of mucopolysaccharides excreted. Also, a group of families was identified in which the mode of transmission of the abnormal trait (mental deficiency) was of an autosomal dominant type. Further attention was also paid to the eighth year in the life history of head-joined Siamese twins which have been separated surgically. These appear without discernible functional involvement at this time.

In a patho-anatomical study of developmental porencephalies and polymicrogyria based on eleven human brains, it seems clear that the gross defects involve mesial and parasagittal areas of both hemispheres, as well as large portions of centro-lateral aspects of the hemispheres, while the entire cerebral convexity may be absent. From the histology, it seems that such lesions may originate prior to the sixth month of gestation and may be related to a common causative factor. Analysis of topographical distribution of the large pallial defects and of the polymicrogyria in the present series suggests arterial origin of these two lesions. In another series of patients, karyotypic examination of 122 persons revealed chromosomal abnormalities of diagnostic importance in 19 percent. Moreover, one set of female twins, one of whom is a mongoloid and has 48 chromosomes, and the other is normal and has 46 chromosomes, was proven to be identical by blood group, protein types and palm prints, despite the discordant trait of mongolism. It is thought that the original zygote was a 47 chromosome mongoloid.

Epilepsy

The clinical and biochemical study of epilepsy in childhood continues and during this period has involved 32 patients. In one group from this series, a 1-leucine sensitive hypoglycemia was discovered. This was satisfactorily controlled and the seizures eradicated by the development of a diet which restricts 1-leu-

cine and is administered in seven divided portions so as to separate principal feedings of protein and carbohydrates.

The ketogenic diet has been re-evaluated in ten children with generalized seizures. Through the detailed study of their lipid profiles, it was found that the average values for the total lipids, total cholesterol and total ketones in the plasma increased significantly by the tenth day of the high fat diet, while increase in plasma phospholipids and triglycerides was less striking. Plasma glucose levels were consistently low when the children were on the diet.

Ninety-three patients with epilepsy have been studied in the operating theater during the course of therapeutic procedures designed for the relief of a convulsive disorder. During the course of single unit recording from epileptic cortex of patients, it seemed that two types of cell units were present: those that discharged synchronously with the epileptic waves, and those that discharged independently of the epileptic waves. In selected patients with severe unlocalized (centrencephalic) cerebral seizures, stereotactically placed depth lesions in the Fields of Forel and the zona incerta have been made, and similarly, lesions have been placed in the amygdala by stereotaxic instrument in certain cases of poorly lateralized cerebral seizures.

In a series of 104 temporal lobectomies, 85 cases fall within the excellent or good therapeutic evaluation groups.

In the laboratory, the anterior mesial thalamus was enucleated by microsurgical technique in adult Rhesus monkeys. Thereafter, the ipsilateral hemisphere exhibited high voltage sharp waves and spikes for as long as nine months. This epileptogenic activity could be exaggerated by the use of intravenous Metrazol, but even with the use of such convulsants was restricted to the side of thalamectomy. By callosal section and mesencephalic division of the ascending reticular system, the epileptogenic activity was restricted to the hemisphere in which thalamectomy was performed. These findings suggest that the anterior mesial thalamus has an inhibitory role and acts so as to raise convulsive threshold.

Involuntary Movements

In the laboratory, an attempt has been made to outline the upper border of the nucleus ventralis lateralis in the primate thalamus by use of a preparation in which the superior cerebellar peduncle was sectioned in two monkeys and one chimpanzee, thus resulting in degeneration which was stained by the Nauta-Gygax method. Reconstruction of the first specimen showed degeneration of fibers extending well forward, probably into the posterior portion of the ventralis anterior as well as the ventralis lateralis. In the anterior portion the degenerating fibers reached as high as the junction of the upper middle thirds of the thalamus height. In addition, template reconstructions of a chimpanzee brain prepared with Marchi method after section of the superior cerebellar peduncle, showed degeneration only in the lower half of the thalamus. Fine material was present in the upper portion of the thalamus. A study of the topography of the potential field about the lateral gastrocnemius-soleus nucleus as fired by antidromic shocks was undertaken in order to further understanding of the magnitude of recordable field as related to implanted depth electrodes.

Twenty-five normal human brains have been imbedded in jeltrate after marking the commissures and preparing for section in the transverse plane and horizontal plane with reference to the intercommissural line. Material of this type serves as a basis for anatomical localization of lines in stereotaxic treatment of involuntary movements.

In the clinic, the fronto-striate arrest response has been studied in 30 individuals. This response was characterized by inability to carry out movement, including speech. It has been obtained in the left hemisphere of 18 persons and the right hemisphere in 12. The response is a relatively high threshold, having a mean stimulus level of 8 ma. Ancillary phenomena consisting of mood change, impaired auditory recall during stimulation, post-stimulation confusion and spontaneous inappropriate speech, and contraversive movement of the eyes and head have been obtained. It seems clear that seizure activity is not a feature of this arrest. EEG studies have also been made

of the effect of lesions in the basal ganglia and thalamus and a comparison of depth and scalp activity during normal and induced sleep has been possible through use of this patient material. Patients with thalamic lesions are subject to intensive psychological testing immediately after coagulation and one year hence.

Cerebral Edema

By means of a standardized method of intracarotid injection of injurious solutes, blood-brain barrier damage, characterized by abnormal extravasation of acid dyes, was produced within one hemisphere of the rabbit, while the other served as a control. Comparative assay of the chloride and water content of the same brain was performed varying the type and concentration of the injuring solute and the time interval between injury and termination of the experiment. Findings from this study indicate that the effect of slight or moderate mercurial blood-brain barrier damage is expressed in significantly decreased chloride content in the hemisphere showing extravascular passage of tracer dyes. This suggests a functional disturbance in exchange mechanisms operating at the blood-brain intephase and not a mere leakage through ruptured blood-brain barrier structures. On the other hand, stronger mercurial injury resulted in the development of edema which was associated with an increase in both chloride and water content.

Unilateral blood-brain barrier injury was produced by an intracarotid injection of mercuric chloride, penicillin, or sodium acetrizoate. This injury was followed by a systemic administration of combinations of two different fluorescent and radioactive tracers: (1) red-fluorescent Rhodamine B labeled albumin with green-fluorescent fluorescein labeled albumin; (2) red fluorescent Evans Blue-albumin complex with FLS; (3) EBA with green fluorescent fluorescein labeled gamma globulin FLGG; (4) RLA with FLGG; (5) sodium fluorescein or FLA with C¹⁴ sucrose; (6) sodium fluorescein or FLA with C¹⁴ inulin; (7) sodium fluorescein with C¹⁴ methyl-*o*-glucose. Combinations of red and green fluorescent albumins showed no separation in the distribution pattern on the damaged side, but simultaneous ad-

ministration of red fluorescent albumins and green fluorescent globulins revealed in numerous instances a distinct separation in distribution of protein tracers on the damaged side. In slightly or moderately damaged areas, numerous blood vessels were surrounded by red fluorescence. The perivascular exudates and globules showed a range of color depending on relative concentration of respective tracers. C^{14} inulin appeared to penetrate in damaged areas more extensively than sodium fluorescein or FLA. An interesting result was obtained in sodium fluorescein- C^{14} methyl- α -glucose combination. Whereas in moderate or severe blood-brain barrier damage, both sodium fluorescein and C^{14} methyl- α -glucose spread intensely from the injured vessels, in very slight blood-brain barrier damage in which no abnormal passage of sodium fluorescein could be detected, there was a distinct inhibition of the normal transport of methyl- α -glucose from the blood into the brain tissue. Most of this experimental work was done in the rabbit, but additional and different studies were carried out in the shark in which lemon and nurse sharks provided by the Lerner Marine Laboratory, were subjected to cerebral and cerebellar lesions produced by cold, heat and chemical substances. All of these animals exhibited a striking resistance of brain tissue to injury. Tissue surrounding the lesion showed no, or very little, evidence of either edema or extravasation of the Evans Blue tracer. When a dose of mercuric chloride far exceeding that used in rabbits for disruption of the blood-brain barrier was used in sharks, there was no evidence of extensive parenchymatous extravasation of the tracer. The alteration of the blood-brain barrier was demonstrable only in the intense staining of the vascular walls with the Evans Blue tracer. Sharks subjected to intraventricular infusion of Evans Blue revealed passage of this tracer into the whole ventricular system, but there was no passage of the tracer into the subarachnoid spaces surrounding the brain and cerebellum.

In another study, the effect of systolic blood pressure on the dynamics of brain edema was investigated by alteration of the blood pressure in cats by means of hyper- and hypotensive drugs administered prior to production of

brain edema. Edema was produced by application of a cooled metal plate to the exposed cerebral cortex. It was shown that the lowering of the blood pressure can almost completely prevent the development of edema.

Brain Tumor

Six patients with glioma and two with meningeal leukemia have received four perfusions each with methotrexate. While the drug is very effective in meningeal leukemia, it is not the drug of choice for intraventricular application.

Language and Memory

In this category, the phonemic aspects of induced dysphasia and normal naming errors have been investigated. The present technique is based on a study in which it was found that the type of initial and final phonemes in the nouns influenced the number of dysnomic errors made by epileptic patients during the carotid Amytal test. Thirty subjects were tested twice in the naming of 72 pictures of objects projected rapidly on a small screen. The greater the number of sounds, or phonemes, in the word, the greater were the number of errors in the object-naming task. The number of syllables, however, was not significantly related to the errors. Word frequency for the correct nouns was also a relevant factor in that objects with more common names produced fewer errors than objects with rarer names. However, the substituted nouns were not of higher frequency than the correct nouns. In addition, when 24 subjects were asked to rate objects for familiarity, their ratings were found to be related to word frequency in the language but not to the errors obtained in the object-naming task. None of the aspects of the errors found with the speeded task with normal people were relevant to the errors observed originally during the carotid Amytal tests on epileptic patients. It seems that Amytal may disrupt some auxiliary component of a system in the hemisphere for speech, while speed may be overload some other component.

A language training system has been developed in which 2,500 words of useful language can be transmitted to a previously naive subject in approximately 75 teaching hours. The

catecholamine, eosinophil and various physiological responses of these subjects have been recorded and programmed. It is intended that this system be used with patients who have undergone right or left therapeutic temporal lobectomy in an attempt to correlate language learning, lobectomy and the side of lobectomy.

Based on animal work completed last year, a test designed for memory mechanisms in man has been completed in this period. This apparatus separates the two factors of delay and complexity and thus permits their independent analysis. It will be used on patients with right or left temporal lobectomies.

Effects of Low Temperatures

It has been shown that freezing cold can produce completely reversible occlusion of blood flow in vessels larger than 2 mm. diameter without significant damage or risk of thrombosis for periods up to 30 minutes. This is true for both arteries and veins.

In another study a relatively selective method for brain cooling was used in conjunction with a system for controlled hypotension in which the animal's circulating blood volume was reduced by 'reserving' the blood within a reservoir of extracorporeal circulation for a predetermined time period. When the need for hypotension was over, the blood was returned to the vital circulation. In this combined methodology, cranial surgery at low temperatures and under a relatively bloodless condition, with recovery of the experimental animals, has been obtained.

Neurosurgical Monitoring

Nineteen normal subjects have been subject to comprehensive recording and the acquired data processed. Sixteen of 24 possible correlations between palmar skin temperature and EEG frequency, as recorded from occipital leads, are significant at the 5 percent level. Correlation between left palmar lateralization and right EEG findings was significant in four out of six subjects at the one percent level. Fifty-one patients coming to operation have been monitored for clinical purposes so that the operative course could be recorded in equipment and observed in progress. Thirteen pa-

tients with 14 thalamic electrodes have been studied, as have six patients with two bilateral cingulum electrodes and nine medial temporal electrodes. Amygdala and uncal electrode stimulation was usually followed by respiratory depression and bradycardia.

A moderate increase in plasma 17-OH-CS levels from 11 to 18 mg.% was observed following amygdaloid stimulation, which is similar to changes observed in the Rhesus monkey. No delayed or prolonged suppression of 17-OH-CS levels as seen in the monkey was observed, however, following hippocampal stimulation in an epileptic patient. It is interesting that normal basal 17-OH-CS levels are observed in these patients, indicating that they are in a relatively unstressed condition at the time of the test.

Microbial Analysis of Neurosurgical Environment

The microbial environment in two architecturally distinct neurosurgical theater situations has been studied, using seven cases in one situation and 20 cases in another as the basis for observation. In the uninhabited environment, counts of one or less organism per four cubic feet of air per two-hour sample were obtained. In an 'active' environment, air counts ranged from a high of 11 to less than one bacteria per cubic foot for a 15-minute sampling period. Of 508 surface cultures and 38 air samples taken during seven neurosurgical cases, no hemolytic coagulase positive *Staphylococcus aureus* was identified. In further studies of 20 additional cases in the new neurosurgical suite, a *Staphylococcus* which was DNase positive and heolytic was identified and eliminated. In addition, these studies were helpful in analysis of the plenum ventilation system, indicating a need for correction because of a defect in current flow.

SUMMARY

The future of the categorical projects is presented in detail at the conclusion of each report. In summation, this Branch has principal tasks in the operating room investigation of patients by means of electrophysiological

techniques. The more sophisticated use of the physiological monitoring system, in conjunction with the data programing, is intended to provide the surgeon with a capability for more precise clinical predictability of individual operating course, as well as correlations of physiological information, some of which are now being made. The human physiological profile thus obtained will be correlated with programmed observations made on the ward unit and the biochemical profile which the Department of Clinical Pathology now obtains. It is hoped that this combined data recording and acquisition system will then be applied to the categorical disease entities under study, such as epilepsy, involuntary movements, head injury, brain tumor, etc. The program in head injury should extend its biochemical support and develop a more sophisticated physical basis. This project, and those in epilepsy and involuntary movements, are, to a large extent, concerned with brain stem mechanisms, and thus a kind of functional coherence is evident as a consistent and common characteristic. It is hoped that as these evolve, significant contributions to knowledge of the human reticular system can be made. The head injured patient is essentially a problem in coma, the epileptic patient a problem in alteration in consciousness, and the involuntary movement patient a problem in relevant, although not identical, thalamic mechanisms. Perhaps through anatomical, physiological, and surgical methodology, some further understanding of brain stem mechanisms in general, and reticulo-thalamic mechanisms in particular, will be forthcoming. Problems in communication and the use of information theory are now at hand, and an increasing use of sophisticated linguistic techniques, as well as data processing methods, will be attempted. The conventional, and ever present neurosurgical problems of cerebral edema, hemostasis, and infection remain as challenging targets. Extracorporeal circulation techniques, hypothermia, study of blood clotting, and specific technical sophistication of design for the reduction of infection will continue as high priority goals. It is hoped that in addition to the newly developed surgical techniques of brain splitting, thalamectomy, etc., the micro-

surgical methods can be extended to cerebral blood vessels so as to approach problems of the intracranial carotid circulation.

OPHTHALMOLOGY BRANCH

As in the past years, the investigations of the Ophthalmology Branch fall into the following main categories; physiology of vision, retinal disease, and the physiology and pathology of the uvea, intraocular pressure, and lens. All clinical projects had their counterpart in laboratory studies. Ramifications of the laboratory studies, in turn, often led to basic levels of research and were extended to work in other organ systems while remaining related to the main problem of visual dysfunction. In several instances detailed investigations of the ocular manifestations of rare systemic diseases, although not programmed, contributed to phenomenological and diagnostic aspects of the disorders. Certain studies on diseases of the cornea and ocular tumors were carried out but played a relatively minor part in the overall program.

From April 30, 1964 until May 1, 1965, 154 subjects (patients and normal controls) were admitted to the nursing unit for participation in the various projects. They accounted for 6,738 inpatient days. Data on the patient census are computed for the specified period since in the preceding report, figures from April to July 1964 were not included and the number of admissions for May and June 1965 cannot be anticipated. In the Outpatient Department 1,622 visits of 534 patients were listed in the records and 1,197 consultation requests were answered in this year. Major surgery was carried out on 26 patients and minor surgical intervention in 47 instances.

Active collaboration with other Institutes and Agencies was gratifying. The help of the following units is greatly appreciated: NCI, NIAMD, NIAID, NHI, and NIMH, Division of Biological Standards, Marine Biological Laboratory in Woods Hole, the A.R.C. Institute of Animal Physiology, Cambridge, England, and Fish and Wildlife Service of the U.S. Department of the Interior.

In the Visiting Scientist Program, Professor Antonio Borsellino from the Institute of Phys-

ics, University of Genoa and Dr. Rudolf Kern from the University Eye Department, Zurich, took part in the research activities, as did Dr. Fritz Baumann on an International Postgraduate Fellowship from Geneva, Switzerland.

Physiology of Vision

Praise is put forth for the effort and the considerable progress achieved by the Section on Physiology in obtaining basic facts necessary for understanding the mechanism of vision. New investigations on electrical interaction among different cells in a single ommatidium of the limulus paved the way for identifying the processes responsible for activating visual receptor cells. The relation between the light stimulus and electrical changes in visual cells was shown to be characterized by two parameters with the dimensions of rates. One of these parameters controls the delay of responses to brief light flashes. Light adaptation reduces amplitude and speed of these responses but the rate of rise is not affected by the state of adaptation. In contrast to this disparity of response patterns, increase of temperature influenced speed and maximum voltage of responses to light flashes in the same proportion. Studies on temperature dependence of reaction rates suggests that a chemical reaction or reactions are operating in the production of visual responses. It is planned to possibly identify the chemical processes required for generation of visual responses. Also attempts will be made to characterize the subcellular structures which are primarily affected by light.

Other microelectrode studies on the limulus in which the light evoked potential changes were recorded simultaneously in the retinal and retinula cells, as well as in eccentric cells, indicated that all these cells are electrically connected. The retinula cells could be the only source of the generator potential and the electrical event may spread to the eccentric cells to initiate the nerve impulse.

Electrophysiological studies on the eye of the honey bee disclosed that the membrane of its visual cell has complex properties; some typical of a nerve membrane and others characteristic of a receptor cell membrane. The first type of property is deduced from the non-linear vol-

tage-current relationship in these responses and the appearance of a fast transient. The second property may be responsible for the decrease of resistance induced by light adaptation. The shape of the responses to a light flash may be the expression of both of these properties.

In the Laboratory of Electroretinography, micro and macro electrodes were used to record electrical responses from the retina *in vivo* to analyze the light induced potentials in this tissue on a cellular level. Responses of various cells in the retina were separated by changing wave length and energy and by controlling the spatial distribution and time function of the light stimulus. A technique was developed to introduce under biomicroscopic observation fine glass micro electrodes in the surface layer of the intact eye of the anesthetized Rhesus monkey. The stimuli consisted of small spots or patterns of monochromatic light. This technique offered a significant opportunity to distinguish three functional type of ganglion cells in the central primate retina. These are, cells with spontaneous high frequency activity which was initially inhibited by light, cells with moderate spontaneous activity initially excited by light and, finally, cells which appear to code color information. The last group is excited by light at one end of the visible spectrum and inhibited by light originating at the other end. In the future, responses of single retina cells will be correlated with their histology. Optic nerves have been sectioned in order to isolate the responses of individual bipolar cells.

In the Laboratory of Cell Biology new physical-chemical studies are directed toward demonstrating the role which light-induced bleaching of rhodopsin plays in the excitation of the visual system. This area of study turned out to be productive and brought forth an array of fascinating facts which cannot be summarized readily. Bleaching of rhodopsin in a monomolecular layer at an air water interface resulted in a slight decrease of surface tension. Retinene deposited underneath the rhodopsin monolayer exerted the same effect. The bleaching effect on the surface tension could be explained by penetration of retinene released from rhodopsin during bleaching into the rhodopsin layer.

Another aspect of the investigation was concerned with cation leakage of rhodopsin in artificial membranes, of rhodopsin micelles, and of isolated rod outer segments after illumination. Only the last method rendered positive results. It illustrated that in the dark there was slow outward leakage of both sodium and potassium from isolated rod outer segments of cattle. Following illumination there was a continuing loss of potassium but accompanied by an equal increase of sodium. Indeed, this effect could be brought about with weak light stimuli (2 seconds, 125 foot candles) which essentially caused no bleaching of rhodopsin. All transretinene in low concentrations (10^{-6}) gave the same results. It is concluded that an ionic mechanism operates in the activation of the photo-receptors on illumination. Bleaching of just one molecule of rhodopsin causes a localized change of permeability of the rod sac resulting in Na-ion influx and K-ion outflow. The cation gradient might be reestablished via the sodium-potassium activated ATPase system. Theoretical calculations and directly observed data on cation movement were in good agreement with each other. It is proposed to strengthen the new theory of rod activation by further investigation.

Preliminary studies on glycoprotein synthesis in the retina have been initiated. Techniques will be utilized by which recently a single enzyme function in the terminal step of biosynthesis of the product was demonstrated. This enzyme is present in various tissues but predominantly in tissues with secretory function. It is probable that the retinal pigment epithelium belongs to these tissues and is active in transferring single sugars to incomplete glycoproteins. This reaction appears to be the final steps in the synthesis of the carbohydrate side chain of these glycoproteins. The glycoproteins in the vitreous humor may originate from glycoprotein synthesis on the retina. With the limited quantity of tissue material from the eye a continuing uphill struggle is anticipated in applying the necessary elaborate methods.

Another approach to solve retinal problems of biochemical nature is an attempt, in the Section of Chemistry, to obtain purified rhodopsin by extraction with a new detergent BC270, alkyl poly- (ethylene oxide) ethanol. When rod

powder was treated with this detergent and analyzed in the analytical ultra-centrifuge, the preparation exhibited two fairly sharp symmetrical peaks, one representing the Rhodopsin-BC270 complex and the other BC270 micelles. By lowering the concentration of the detergent the preparation approached a purity that resembled that arrived at by digitonin extraction. Chromatographic purification was easier on the BC270 rhodopsin material. Obviously it has to be shown first to which degree purification can be accomplished in order to steer clear of contaminations before difficult physical-chemical examinations are carried out.

Retinal Disease

Efforts expended in studies of degenerative diseases of the retina have been remarkably productive. New observations were numerous and trustworthy in view of the elaboration of methods of psychophysical and electrophysiological testing. More precise information became available on the functional disturbance in rare disease entities such as congenital stationary night blindness, fundus flavimaculatus, and acanthocytosis (a-beta-lipoproteinemia). The orderly investigation of the systemic disease, acanthocytosis, represents a first-rate cooperative effort which led to the major discovery that, in this form of tapeto-retinal degeneration, treatment with vitamin A in soybean oil improves retinal function. An achievement of practical significance was the refinement of static perimetry to permit recognition of loss of retinal function in certain retinopathies, such as that produced by chloroquine intoxication, at an early stage when other techniques fail to be of diagnostic value.

To this group of investigations on the retina belong experimental attempts to produce deterioration of the visual receptors by continued exposure of rats to light and by inducing accumulation of abnormal fatty acids by chronically feeding tetramethylhexadecanoic acid to rats. No results have been obtained so far in this study.

A multidisciplinary clinical approach in the study of vascular retinopathies has still not provided leads for the understanding of pathologic events resulting in severe visual loss.

Neither routine laboratory testing nor hematologic, immunochemical and chemical (lipid), or histologic examinations of skin muscle biopsies turned up useful information as to the etiology of the disease.

Most recently workers in the Laboratory of Perinatal Physiology observed that young monkeys with experimental allergic encephalomyelitis (EAE) developed eye pathology. The histologic examination of eight eyes from this group of animals showed, in most instances, pathology of the retinal vessels or hemorrhagic conditions varying from a mild to a most destructive degree. The histopathologic involvement of the optic nerve was not related to the severity of the retinal lesions. These new findings are of interest since certain facts suggest that EAE is of allergic nature and that autoimmune mechanisms may be at work in some of the human vascular retinopathies accompanied by recurrent hemorrhages. It will be necessary to accumulate more material and to follow the developments of the ocular changes. If such material becomes available, it will be instructive to carry out electron microscopic work.

Uvea

Patients with uveitis of various age groups represent the greatest number of in-patients. Results of corticosteroid therapy and chemotherapy combined with hospitalization were satisfactory in a great number of the patients, but the etiology of the disease remained obscure as a rule, or was considered suggestive at best.

The use of Methotrexate (MTX) was continued in a selected group of patients with anterior uveitis. The most dramatic improvement was observed in a patient with sympathetic ophthalmia, whereas cases with cyclitis responded slowly but by and large favorably. Behcet's disease appeared refractory to the therapy. Immunologic studies on these patients supported the concept that MTX does not suppress delayed hypersensitivity and secondary antibody responses, in contrast to the inhibition of the primary antibody response and of the mononuclear phase of the inflammatory reaction as tested on the skin. The study will be extended to more patients in whom other forms

of therapy have proven to be ineffective and to investigation of the advisability of long-term anti-metabolite treatment.

In an experimental study ischemia of the choroid was produced in the cat by injection of a thrombosing agent into a vortex vein. With this technique areas of the choroid vascular bed are eliminated whereas circulation in the retinal vessels is not altered. Thus the role of the choroid in nutrition of the retina can be studied under most favorable conditions. It could be demonstrated in histologic examination that the severe ischemia of the choroid produced severe damage of the photoreceptors and their nuclei and also of the pigment epithelium. Minimal ischemia caused injury solely to the cones and rods and the pigment epithelium. The experiments proved directly the dependence of nutrition of the outer layers of the retina on the choroidal circulation. Chronic experiments utilizing these new techniques are in progress.

Intraocular Pressure

In the last year the glaucoma project has been greatly expanded, both in its clinical as well as its laboratory phase. Aqueous humor dynamics and hemodynamics in diseased human eyes and in experimental animals were the subjects of several studies.

In a clinical investigation effects of topical administration of corticosteroids on aqueous outflow were re-examined by critically studying the influence of the steroids on the responses to a water load. Behavior of the normal and glaucomatous eye in the water drinking test, and effect of rapid changes in the arterial and venous pressure on the intraocular pressure were analyzed, and new methods were employed to study the interrelationship between autonomic nervous system activity and the intraocular pressure. An array of various techniques served in the different phases of these examinations.

The rise of the intraocular pressure in response to topically administered corticosteroids was rapid in familial open angle glaucoma, slower in secondary glaucoma, and delayed and slight in non-glaucomatous eyes. Responses to the water drinking test were definitely patholog-

ical when the steroid-induced pressure rise was large, but lowering of the C value was questionable in eyes with a slight response to steroid provocation. These findings are pertinent to the current controversy over the mode of action of corticosteroids on the outflow mechanism.

The relation of serum osmolarity to the rise of IOP in the water drinking test was thoroughly studied, and it was concluded that the osmotic effects are not the only cause for the increase of IOP following ingestion of a large volume of water. It is not clear, however, which other mechanisms come into play in causing this pressure rise.

Different techniques were used to compare the responses of the systemic venous pressure and the IOP to increased intrathoracic pressure. Elevation of the intrathoracic pressure is followed by a slow rise of the venous pressure and a much more precipitous increase of the IOP. A certain independence of the two functions was demonstrated by varying the methods of inducing changes of the intrathoracic pressure. Glaucomatous eyes responded to this test in an irregular fashion.

Patients with glaucoma and normal controls did not exhibit any correlation of changes of the IOP and those of other autonomic functions such as skin resistance and pulse rate.

Throughout these studies it was obvious that the repeatability of tonographic measurements leaves much to be desired. Certainly this technique does not simply measure the mechanics of outflow. Its results are influenced by vascular, nervous, and possibly hormonal factors in addition to mechanical considerations. Future examinations may show whether the influence of these factors on the IOP can be defined so that they could be made the target of therapy.

The regulation of IOP by various factors particularly by reactions of the intraocular vasculature remained the main area of research in the Section on Pharmacology. Enucleated arterially perfused human and cat eyes were studied to delineate their usefulness as test objects for pharmacological and physiological studies. An ERG could be elicited from isolated eyes despite elimination of normal blood supply and nerve connections. The persistence *in vitro* of such a sensitive response

increases the value of these preparations for pharmacological and physiological investigations.

C^{14} -labeled inulin was used to measure the aqueous humor turnover rate in cat eyes and this technique was applied in a series of experiments. It was established that in isolated eyes the rate of aqueous turnover was 35% lower and anterior chamber volume was 15% smaller than in eyes *in situ*. Reinvestigation of the effect of acetazolamide with this method demonstrated that, in small doses, the drug lowers IOP without decreasing the rate of aqueous humor formation. Much higher doses are required to achieve the latter effect. These results are in agreement with the concept that, under the experimental conditions, acetazolamide decreases IOP by a vascular mechanism. The hypotensive response induced by ouabain was also examined, and in this case, a dose-dependent decrease of aqueous humor formation was observed.

In conjunction with these experiments, the effects of acetazolamide and epinephrine on vessels of the isolated lateral chorioplexus of the cat were investigated. The technique of perfusion of the chorioplexus was the same as that used in isolated iris preparations. Acetazolamide and nor-epinephrine caused vasoconstriction of the plexus vessels but not of the cerebral vessels, whereas eserine, ouabain, histamine and serotonin had a constrictor effect on both the plexus and brain vessels.

The effects of betamethazone, a compound which increases the IOP in man on chronic local application, were examined on the vasculature of arterially perfused irides. It appears to act as a smooth muscle depressant and prevents the vasoconstriction induced by eserine, ouabain, arterenol, and acetazolamide. A rise of IOP following betamethazone perfusion was also shown in the enucleated eyes.

In another study an attempt was made to explain the nature of the uniform vasoconstrictor response of the iris artery to sympathomimetic and parasympathomimetic agents as well as to histamine, ouabain, and acetazolamide. From the analysis of the results obtained with the use of blocking agents it was concluded that the parasympathomimetic agents act by stimulating cholinergic post-ganglionic receptors

whereas the catecholamines appear to stimulate post-ganglionic sympathetic receptors. The action of ouabain and acetazolamide may be caused by direct stimulation of smooth muscle fibers. The strange similarity of the responses of the iris artery to pharmacological antagonists is not explained by the results of this study and require further investigation. Work on the corticosteroids has to be expanded in view of the great practical interest in the ocular effects of local steroid therapy.

A bioassay, previously designed for analysis of the effects of pharmacologic agents on muscle strips of iris and ciliary body, proved of great value in the study on the action of corticosteroids on these muscles. It was clearly demonstrated that betamethazone depresses alpha receptors of the sphincter and the dilator of the iris and of the ciliary body. Very low concentrations of the drug reduced greatly the effect of acetylcholine, 1-epinephrine, 1-norepinephrine or serotonin. The alpha receptors of the sphincter appeared to be more sensitive to the effects of low doses of betamethazone than those in the dilator. The negative myotropic effect in the strips of intraocular smooth muscles has significance in the interpretation of steroid induced intraocular pressure rises. Smooth muscles other than those of the eye will be tested for comparison.

Studies on the extraocular muscles represent a new area of investigation in this Branch. Again use was made of the techniques previously devised for intraocular muscle strips. It was discovered that in the superior rectus muscle of the rabbit slow and twitch fibers form individual muscle plates which can be separated from each other by careful dissection. Histologic examination indicated that there is practically no mixture of fibers in the two layers. The slow fiber preparation responded to acetylcholine with tetanic contractions whereas the twitch fibers which make up the main bulk of the muscle were either refractory or showed only transient and slight responses. The latter could be explained by the presence of a few slow fibers in the twitch fiber portion of that muscle. Experiments provided direct evidence that the slow fibers maintain the muscle tone. It is hoped that electrophysiological

studies on single twitch and slow fibers can be conducted in the future.

Another phase of this study was concerned with the distribution and nature of adrenergic receptors in extraocular muscles in cat, monkey and rabbit. It was shown that the recti and oblique muscles of the rabbit and cat have alpha and beta receptors whereas in the corresponding muscle of the monkey only beta type receptors were demonstrated. It is probable that the slow fibers in the superior rectus of the rabbit carry the adrenergic receptors. The study will be continued on muscle of other species.

Cornea

Infectious and degenerative diseases are being continuously investigated, particularly with regard to fungus infections, a project which was included in last year's report.

In the Section on Chemistry, corneal collagen was submitted to extensive physical-chemical studies. The molecular weight, the molecular dimensions, and the helical structure were similar to those of other collagens, but differences were noted in the amino acid composition and in the temperature range for helix to coil transition. A collagen has been isolated which not only appears to be monomeric, but has minimal intramolecular cross-linking. In contrast to the very narrow transition zone of the polymeric corneal collagen, the monomeric has a transition zone of a width characteristic of collagen of other sources. This implies that the structural stability of the polymeric form can be attributed to intramolecular links between the subunit chains and intermolecular cross-linking between monomeric collagens forming the polymer. These findings might be of functional significance. The data provides a base for comparative studies of different collagens of the eye and for studies of the role of collagen in corneal development, and in corneal pathologies. Studies of such material are in progress.

Lens

The activities of the Section on Cytology and Histopathology have been oriented primarily

toward problems of cataractogenesis. In histological and histochemical studies on lenses obtained from the operating room no changes have been observed which would throw light on the mode of action of certain cataractogenic agents such as MER-29 or cortisone or on the significance of systemic diseases such as agammaglobulinemia or local diseases such as uveitis for the development of lens opacities.

An interesting new experimental cataract has been produced in young trout by long-term feeding of thioacetamide, a compound known to produce hepatomas in rats. Biomicroscopically, the opacity appears to originate at the anterior pole and progresses to engulf most of the lens cortex. Histological examination demonstrated a circumscribed proliferation of the lens epithelium at the anterior pole. The cells here seemed to invade the underlying cortical fibers. It, as yet, undetermined whether the active proliferation of epithelium is the initial event or whether this proliferation is simply a response to a cortical lesion. The question is of considerable interest in view of the carcinogenic nature of thioacetamide and the fact that cancer of the lens has never been observed. It is planned to produce this cataract in rats, a more convenient experimental animal, so that studies of the developing opacity and of induced cytologic changes can be more carefully controlled.

Studies on the normal population dynamics of the lens epithelium and of the influence of certain cytotoxic and cytostatic agents on the cell system have been continued. Investigations on the antimitotic action of the alkylating agent, triethylene melamine was extended to an examination of the effects of the drug on DNA synthesis as determined by incorporation on H^3 -thymidine and autoradiography. From the observations made in this prolonged study, it is concluded that triethylene melamine significantly reduced the rate of DNA synthesis and that its mechanism is markedly different from that of a previously studied alkylating agent, Myleran.

Investigations of the action of Myleran were continued in a study of the effect of chronic administration of the drug and, as expected from the postulated mechanism of action of the com-

pound, profound depletion of the epithelial cell population was observed. This accounts for the known cataractogenic property of myleran in animals.

Continuing efforts to establish the pattern of normal population dynamics in this cell system as a prerequisite for studies of toxic agents led to experiments on the diurnal variations in mitosis and DNA synthesis, and the effect of age on these rhythms. Preliminary results demonstrated that the extent of moderate fluctuation of mitotic activity observed in young animals does not appear to change with age, although there appears to be a time shift of the periods of maximum and minimum activity. Corresponding to the variations in mitotic activity there is a marked fluctuation in the number of DNA synthesizing cells of nearly the same magnitude, but approximately 12 hours out of phase. This phase difference may be explained by the timing of events in the cell cycle in which the initiation of DNA synthesis precedes mitosis by about 12 hours.

Systematic work in the laboratory of electron microscopy had to be interrupted because the head of this unit has not yet been replaced. The facilities of the laboratory have been used in connection with histologic studies in a few instances but basic research had to be postponed until the position of the head of the laboratory is filled. However, during this time some technical improvements on the electron microscope have been accomplished.

Ocular Changes in Systemic Disorders

In this report year cooperation with investigators of other institutes was particularly fruitful in studies of systemic diseases in which involvement of the eye played an important part. Reference has been made to the discovery of the beneficial effect of Vitamin A treatment on the Tapeto-Retinal degeneration which accompanies acanthocytosis, a systemic disorder characterized by the absence of a beta-lipoprotein. New ophthalmologic observations were described in other disease entities. In fifteen patients with carcinoid disease, prodromal signs, such as severe lacrimation and injection of conjunctival vessels presaged the onset of a true flush. Retinal changes consisted

either of multiple retinitic spots in the macular area or exudative lesions in the periphery, in addition to punched out choroidal atrophic foci. The latter may have developed from the exudative process. They are thought to be connected with vascular pathology developing during the characteristic attacks.

In six children with cystic fibrosis of the pancreas, various stages of optic nerve involvement were encountered, ranging from retrobulbar neuritis of moderate degree to total atrophy of the optic nerve. Histological examination demonstrated degeneration of the retinal nerve fiber layer and demyelination and loss of axon cylinders in the optic nerve. It is generally accepted that this nerve pathology is produced as a toxic side effect of prolonged chloramphenicol therapy.

15 members of a family, in which the Ehlers-Danlos Syndrome was inherited, exhibited the characteristic manifestations of the disorders. Ophthalmoscopic examination disclosed as a new finding the presence of angioid streaks in the propositus and her daughter without signs of pseudoxanthoma elasticum.

Finally, 39 members of two families in which Fabry's disease (a glycolipidosis) occurred were studied ophthalmologically. In addition to previously described ocular manifestations of this disease a characteristic delicate cataract was observed for the first time. Genetic studies support the concept of sex linked transmission.

ELECTROENCEPHALOGRAPHY AND CLINICAL NEUROPHYSIOLOGY BRANCH

Clinical-Diagnostic Service

This service represents a considerable portion of the overall activity of the Branch, over 30% of man-years being devoted to it.

From March 31, 1964 to March 31, 1965 a total of 1,749 examinations have been carried out in the EEG Laboratory. These examinations were performed on both in- and out-patients (1,345 and 404, respectively), following referrals from the various Institutes in this order:

Institute	No.	%
NINDB	1,025	58.5
NCI	216	12.3
NIMH	174	10.0
NIAID	139	8.0
NIAMD	107	6.1
NHI	88	5.1
Total	1,749	100.0

The monthly average of EEG examinations (145) has slightly increased from that of the last report but remains fairly close to the monthly average for the last 6-7 years and to what appears to be a resonable figure, considering the available facilities, the type of examination that is generally required and the needs of the various Institutes.

Most of these examinations are performed as a clinical service or as part of investigative projects in which this Branch (or Institute) has no direct interest; some of this activity, however, is also applied toward the fulfillment of projects in which this Branch cooperates and/or is primarily involved (see description of individual projects). This is true, for instance, in the case of the numerous studies (see Branch of Surgical Neurology) carried out in patients in whom electrodes have been chronically implanted for diagnostic-therapeutic purposes. In addition to these and to the routine EEG scalp examinations, records have been obtained directly from the exposed cerebral cortex in the course of 27 surgical operations.

Research Activity

Some of the projects already outlined in previous reports have been completed in the period covered by the present report. Among these the results of the microelectrode analysis of the nucleus gracilis of the cat were presented at the annual meeting of the American Electroencephalographic Society (Santa Fe, New Mexico, October 1964) and at that same meeting, this report was awarded the Hans Berger Prize as the best paper from a junior investigator. Among its findings are: a) the identification and distinction of pre- and post-synaptic elements in the nucleus; b) the relative specificity in the topographical (rostro-caudal) distribution of neurons responding to different sensory modalities; c) the relationship between extent

of nuclear receptive fields and peripheral areas; d) the powerful influence (mostly "inhibitory") of the motor cortex upon the activity of over 50% of the elements of the nucleus and the suggestive topographical arrangement of these elements in relation to their different susceptibility to such corticofugal effects; e) the anatomo-functional distinction apparently existing between different portions of the nucleus.

The findings of another study related to project 1001(c) have also appeared recently in printed form. The project is related to one of the experimental forms of epilepsy, and deals with the effects (and their mechanism) of local cortical strychninization on various types of inhibitory post-synaptic potentials in neurons of the cat motor cortex. On the basis of suggestive results it has been possible to offer an alternative to the commonly accepted hypothesis. Thus strychnine, rather than interfering with the action of the inhibitory transmitter, could actually damage the neuronal membrane and, consequently, abolish the post-synaptic hyperpolarization or even convert it to a depolarizing potential.

A new project underway is the study of the electrographic correlates of the various phases of nocturnal sleep. This project involves the time-consuming procedure of collecting data during the entire night while selected patients, with a number of intracerebral electrodes, are in their natural sleep. It has long been recognized that the electrical activity of the brain goes through a series of different patterns which are apparently related to the different "depths" of sleep. More recently a sleep phase has been identified with well defined clinical and electrographic characteristics. This phase has been, and is currently the object of different forms of investigations in both animal and man. It is also a well known fact that various seizure disorders are variably influenced by the sleep-wakefulness cycle and that certain forms of epilepsy tend to be "activated" during sleep. The present patient material lends itself to the interesting study of this general problem of sleep in "normal" and epileptic subjects. To date no definite results are available but the collection of data and their analysis continues

and it should soon be possible to learn something about the role of various subcortical structures in, and their activity during, sleep as well as the possible mechanisms of nocturnal seizures.

Other projects have been started in the course of this fiscal year and deal with specific problems about the physical properties and the basic physiology of neurons. These projects are at different stages of investigation and none has been completed at the time of this report although the experimental phase of the work is over. Briefly, the main purpose is to obtain information about resistance, time constant and rectification properties of the membrane of neurons in the cat cerebral cortex. These data are studied in resting conditions and during synaptic impingement in the "normal" neurons as well as in neurons which have been affected by local strychninization. It is hoped that some of the data might help to understand the nature of the so-called spontaneous activity of the brain.

The analysis of the experimental data from these studies involves a long series of careful measurements and calculations. These are presently in course and the major findings which are mentioned in the project descriptions should be considered provisional.

A critical survey has been completed of the world literature in the field of epilepsy for 1964 (as well as the 1963 material which had not been included in the analogous study of the previous year). This review will form the chapter on "Epilepsy" for the yearly volume of "Progress in Neurology and Psychiatry". Over nine hundred papers dealing with various aspects and forms of seizure disorders, experimental convulsions, their experimental and clinical pharmacology, etc., have been surveyed. Besides the direct interest of both Branch and Institute in this field, this modest effort has required a relatively small investment in man-years and should prove of some practical usefulness in an era of over-publication which greatly hampers any individual attempt at bibliographic documentation. A theoretical-critical discussion has been published on the nature of the electrical activity of the brain. The various experimental data which

might throw some light on the possible relationship between neuronal activity and "slow" waves of the EEG, have been analyzed and discussed.

Other Activities, Organizational Aspects and Miscellaneous Information

Training in clinical electroencephalography has been provided for two doctors. The type and extent of such training is felt to be adequate for a neurologist primarily interested in clinical work and simply wishing to broaden his practical knowledge in one related laboratory technique but it could hardly be considered sufficient for a person who plans to become a full-time, Board qualified electroencephalographer.

Some of the reasons which are more or less directly responsible for the limited usefulness and/or unsatisfactory results of this important part of this Branch activity have been outlined in the previous reports; but nothing could be done to modify the existing situation and, whatever the achievement, these are almost exclusively due to the efforts and efficiency of the technical staff and of the medical officer of the Branch.

Space remains a serious problem in the clinical Section of the Branch and a particularly acute one for the professional staff which has to work in rather unfavorable physical conditions. The same problem has also prevented the creation of a record library, an important facet of all EEG Laboratories. Due to lack of conveniently located storage space, records older than two years have to be microfilmed and are subsequently destroyed. The microfilm has limited usefulness and a considerable amount of interesting material is no longer available.

The Branch Chief has continued his part-time activity as Chief Editor for American and the Far East of the International monthly Journal, "Electroencephalography and Clinical Neurophysiology". This challenging and interesting form of activity is considered rather rewarding and it is an activity which greatly contributes to the overall scientific standing of the Branch and, indirectly, of the Institute.

The Branch Chief is presently preparing the material for a number of symposia and discus-

sions in which he has been officially invited to participate next September, on the occasion of the various International Congresses to be held in Vienna and Prague. Details of these presentations will be outlined in the next year's report.

The official positions of the Branch Chief include that of Delegate for the American EEG Society to the International Federation of Societies for Electroencephalography and Clinical Neurophysiology and that of Chairman of the Board of Qualification (in clinical electroencephalography) of the American EEG Society. He has recently been nominated to succeed Dr. M. A. B. Brazier as President of the International Federation of Societies for Electroencephalography and Clinical Neurophysiology but has refused this honor in view of the excessive time and activity this office would involve.

The Chief EEG Technician, Mrs. M. Berkeley, was chosen to be the second President of the recently formed American Society of Electroencephalograph Technicians, an honor she fully deserves.

Future Programs

The main investigative interests of the Branch will not shift significantly from those outlined in the present report.

Due to the particular organizational position of this Branch, projects of a primarily clinical nature have to depend almost entirely on the available patient material, or, in other words, they are determined by the current research interest of other Branches and Institutes. In this respect, a close and reciprocally beneficial cooperation is anticipated to continue with the Branch of Surgical Neurology of our Institute.

A certain amount of modernization of equipment is considered. This should require a slight transitory budget increase but also permit a broader or different technical approach to various problems related to the electrical activity of the brain in normal subjects and patients with CNS ailments. Epilepsy in its different aspects and manifestations, and especially its pathophysiology and diagnosis will continue as one of the main areas of investigation in both clinical and experimental fields. Neurophysi-

ological research will probably remain centered around cortical phenomena and/or subcortical-cortical integration in mammals.

LABORATORY OF NEUROANATOMICAL SCIENCES

Introduction

During FY 65 the four Sections of the Laboratory of Neuroanatomical Sciences have undertaken a total of 30 projects, most of which have been brought to completion, and many of which have yielded results which form the basis of new projects which are now being undertaken. Every major segment of the nervous system has received attention. A balance has obtained in the attention given to the major sensory systems, the central nervous system, and the efferent pathways and neuromuscular systems. Investigation has been conducted at the gross, microscopic, and ultrastructural levels. It is especially true in the nervous system that a purview of organizations at all of these levels is essential to a complete understanding of the patterns of neural organization which form the morphological basis of function. In a program of this size the selection of the most suitable species for the study of a given phenomenon has quite naturally led to the use of vertebrate forms drawn from all five classes. The occasional use of the comparative approach has also tended to assure the use of a wide range of species in the work of the laboratory. During the past several years the interest in developmental phenomena such as cytotogenesis, histogenesis and morphogenesis, which has always been a concern of this laboratory, has been slightly expanded and has come into balance with the other approaches used in the laboratory. The output of the laboratory during the past year can be summarized most succinctly by discussing each of the major segments of the nervous system in turn.

The Sensory Systems

Input to the nervous system occurs through a restricted number of sensory channels, each of which transduces qualitatively different type of information from the environment and en-

codes this information in such a way that it can be transmitted in the form of neural impulses to appropriate stations in the central nervous system. The Laboratory of Neuroanatomical Sciences devotes about half of its resources to an analysis of the structure and organization of the sensory input pathways. Each major pathway is under analysis at every level from the peripheral sense organ to the terminal connections which are made centrally. Structural analysis is carried out from the gross morphological level through the level of fine structure as revealed by the electron microscope. In many instances comparative analysis of the sensory systems is undertaken using representatives of each of the five vertebrate classes. Increasing attention has been focused on the sequence of individual steps which, during embryonic development, are responsible for the cytotogenesis, histogenesis and morphogenesis of individual parts of each of the major sensory systems.

The Auditory System

A battery of techniques (Golgi, Koelle, Marchi, Nauta-Gygax, Rasmussen, electron microscopy, etc.), coupled with experimental lesions placed at different levels of the auditory system of adult mammals (cat, chinchilla, primates) are revealing in increasing detail the complex interconnections that occur between the several levels of the auditory system, as well as between the auditory system and other portions of the central nervous system. During the past year the following specific findings have resulted: 1. A centrifugal pathway has been identified which connects the superior olive to the ventral cochlear nucleus. This pathway terminates in a plexiform synaptic arrangement on the cells of the ventral cochlear nucleus in conjunction with afferent caliciform endings of the cochlear nerve; 2. The discovery of a pathway connecting the nucleus of the trapezoid body with the homolateral superior olivary nucleus, coupled with the centrifugal pathway mentioned above, provides a missing link in the chain of neurons that interconnect the cochlear nuclei of both sides. Knowledge of this pathway offers an explanation for physiological data which until now has had no mor-

phological basis; 3. The relative population of afferent and non-afferent synapses on the cells of the cochlear nuclei is different in different regions. The vast majority of the synapses terminating on the cells of the dorsal cochlear nucleus, as well as of the anterior one-third of the ventral cochlear nucleus, are of the non-afferent type. Most of the terminals on cells of the posterior two-thirds of the ventral cochlear nucleus are of the afferent type; 4. Changes in the cells of the cochlear nuclear complex are consistent with the notion that the degree of transneuronal atrophy is dependent upon the number or proportion of endings which have been destroyed by deafferentation; 5. Following deafferentation, the number of residual non-afferent type endings increases during the first post-operative month. This increase is possibly due to sprouting of non-afferent terminals, and raises interesting possibilities concerning dynamic balances which may exist between populations of different types of terminals on the surface of the same neuron. Recently investigations were begun with the electron microscope to explore fine structure, and fine structural changes, of the ventral cochlear nucleus following destruction of the spiral ganglion. 6. The medial geniculate body, which lies at a higher level in the auditory system, has a neuronal architecture in the cat which permits its division into three nuclei: medial nucleus; ventral nucleus with a laminar arrangement which may reflect a tonotopic organization of the fibers representing the cochlear input; dorsal nucleus which has a mixed input originating, in part, in the region of the midbrain medial to the brachium of the inferior colliculus, and, in part, from fibers that leave the optic tract. The dorsal nucleus may thus have an integrative function. This analysis has opened the way for a more meaningful physiological analysis of the medial geniculate body.

Experiments have been performed, and are now awaiting analysis, which will extend the types of findings previously made on lower mammalian forms to the primates.

The cytogenesis and histogenesis of the inner ear is under analysis in chick and mouse embryos. By injecting tritiated thymidine at known stages in development, and by subse-

quently analyzing the distribution of label in the cell nuclei of the vestibular and cochlear portions of the inner ear, the step by step sequence by which these organs are formed in being established. One base lines have been established it is planned to undertake similar analyses of a series of strains of mice, each of which bears a single mutant gene which interferes with one or another step in the developmental sequence. It should be possible to identify the time of onset and the nature of genetic defects, some of which are quite similar to congenital defects of the inner ear seen in man.

Olfactory System

Light and electron microscopic investigation of the olfactory mucosa revealed two types of cells: bipolar olfactory neurons, and supporting cells. The dendrites of the olfactory neurons have the following properties: 1. They are up to 200 micra in length; 2. their shafts contain single fibrils throughout most of their length; 3. vesicles lie between the ciliary membranes and the ciliary fibrils of the shaft; 4. divisions in the shaft which are bridged by double membranes occur with relatively high frequency; 5. the complex system of centrioles and basal bodies which exists at the dendritic apex of the bipolar cell suggests the possibility that the olfactory cilia are continually replaced rather than persisting as relatively permanent structures. In contrast the supporting cells have microvilli on their free borders, and contain granules which suggests that they are the source of mucous material which is discharged onto the surface of the olfactory epithelium. These analyses have provided detailed knowledge of the fine structure of the probable primary olfactory transducers.

The analysis of the fine structure of the olfactory system was extended into the olfactory bulb of the rat with the following findings: 1. The major pathways in the superficial layers of the olfactory bulb were identified at the fine structural level; 2. synapses in the various layers were studied in detail. Dendro-dendritic synapses were discovered. In addition, different synapses between the same two dendrites were shown to be morphologically polarized in opposite directions. These findings are

of some importance since they reveal a new type of synaptic relationship between neurons, and which has already been useful in explaining electrophysiological data; 3. small periglomerular neurons were found to give rise to sheet-like processes which engulfed other periglomerular neurons.

Visual System

One program of the laboratory investigates the mechanisms which control the step by step development of the tissues of the vertebrate eye in such a way that they have sizes, shapes, positions, orientations and differentiated properties appropriate to optical function. It has been possible to identify many steps in precisely timed series of interactions among the tissues of the eye, to specify which tissues are sources of specific influence, and which tissues are targets of influence; to determine the stages of development during which specific influences are mediated. As a consequence we have built a flow sheet which not only explains the orderly morphogenesis of this organ, but also provides a rational basis for understanding many of the defects that result from the interference with one or another step in its development.

A few of the specific findings of the past year follow: Last year work in this laboratory demonstrated a mechanism which operates to continuously control the orientation of the developing lens. The neural retina is the only tissue of the eye which has yet been shown to have a specific influence on the growth of the lens. The continued presence of the neural retina is essential for the normal growth and shaping of the lens. Work this year has revealed that this mechanism is not species, class or age specific, and that it consists of a water soluble, diffusible factor or factors arising from the neural retina. Lens tissue removed from the eye environment has only a limited capacity for autonomous differentiation. Explanation of embryonic lens epithelium into defined or supplemented culture medium revealed that these cells are initially capable of partially differentiating into lens fibers in protein supplemented medium, that this ability is lost when cells are cultured in defined medium

for 48 hours, and that lens epithelial cells which have been maintained in defined culture medium for 48 hours are still capable of forming lens fibers when returned to the environment of the eye but not when they are implanted in the coelom. In addition, it has been demonstrated that lens epithelial cells begin to divide in culture only after they have lost their ability to differentiate in supplemental media. These results are of importance since they have made available an *in vitro* test system which is capable of detecting the presence of the retinal-lens factor. This opens the way for the isolation and identification of this factor or factors.

Further attention has been given to the interactions which occur between the neural and pigmented portions of the retina during development. Neural retina has been successfully regenerated in patches from the pigmented epithelium of the embryonic eye of the chick. This regeneration occurs when the neural retina is separated from the pigmented epithelium by a discrete distance. The pigmented epithelium will regenerate neural retina only under the influence of partially differentiated neural retina or otocyst epithelium. Other neural epithelia (e.g. optic tectum) will not support this regeneration. This study indicates the mechanism which assures continual apposition of the neural and pigmented portions of the retina during development (demonstrated by previous work in this laboratory) has, as one of its functions, the assurance that the pigmented epithelium will differentiate in the appropriate direction, and not form ectopic neural retina. In addition, the way is open for studies into the nature of the factor or factors arising from certain neural epithelia which will elicit regeneration of neural retina from the pigmented epithelium. A test system has been developed which is capable of detecting the presence of such a factor or factors.

Another specific tissue interaction has been demonstrated between the lens and the cornea. The presence of the lens is as necessary throughout the major portion of embryonic life to support the differentiation of the cornea. This finding makes it clear that the induction of the cornea by the lens is not mediated solely during a brief period early in development, but

that this influence is necessary for the continued and orderly development of the cornea. An *in vivo* test system has been developed which may make possible the isolation of the factor or factors arising from the lens which influence the cornea.

Several clinical studies have suggested a higher incidence of lens cataracts among the offspring of diabetic mothers than occurs in the general population. We have accumulated experimental evidence that elevated concentrations of sugar (glucose, galactose, or xylose) in the embryonic blood stream at high enough levels and for a sufficiently long period during development, results in the production of lens cataracts in 100% of the cases. Upon returning blood sugar to normal levels, it was shown that damaged lens fibers underwent complete dissolution followed by removal of their products so that, after a period of time, the lesion was no longer visible, although the arrangement of fibers in the lens indicated the former location of the lesion. Only d-sugars were capable of bringing about these changes, and l-sugars proved ineffective in this respect. These findings emphasize the importance of maintaining careful control of the maternal blood sugar level during pregnancy. If experience in the chick embryo can be extrapolated to the human, elevated blood sugar levels would be much more harmful during the early stages than in the later stages of development.

A study of the development of the higher visual centers (optic tectum in the chick embryo) indicates that the retinotopic map in this neural epithelium develops in complete independence of the ingrowing optic nerve fibers. Thus, separate "maps" appear to develop at the retinal and tectal levels prior to the time at which the corresponding areas of both structures become interconnected by the axons of the ganglion cells of the retina. It is planned to expand these studies during the coming year to determine the sequence in which the neurons of the neural retina on the one hand, and the optic tectum on the other, become electrically active as embryonic development proceeds. In addition, we will determine the mitotic history, site of origin, and pathways of migration of the several neuronal components of the retina and

optic tectum during the course of their development. Labelling with tritiated thymidine, followed by autoradiographic analysis, will be used in this investigation. Experiments have been designed to evaluate the extent to which the several levels of the visual system influence each other.

Attention has also been given to the neurological basis of color vision in fish. Removal of the anterior telencephalon did not result in a loss of color discrimination or generalization as tested in animals conditioned to respond to specific colors and brightnesses. Animals with posterior telencephalic lesions were able to discriminate the training stimuli but could not generalize either from the brightness or color characteristics of these stimuli.

Neuromuscular Relationships

The principal output of the nervous system is to the several types of muscle that comprise the motor systems of the body. The Laboratory continues to explore the fine structure, the mechanisms of synaptic transmission, and the trophic interactions associated with the myoneural junctions of both striated and smooth muscle.

The relatively recent discovery of both granular and non-granular vesicles associated with nerve endings in smooth muscle has given rise to speculation concerning the role of these structures in synaptic and neuromuscular transmission. The finding of de Robertis and Iraldi that monoamine oxidase inhibitors cause a profound increase in the numbers of granular vesicles in the sympathetic nerves of the rat pineal body could not be confirmed for similar nerve fibers in the vas deferens of the rat. Paragiline caused a rise in vas deferens norepinephrine of about 25%. While this was accompanied by an increase in the total number of vesicles per unit area of axon, there was no significant rise in the numbers of granular vesicles. A similar study in the nucleus dorsalis raphe likewise showed no increase in the numbers of granular vesicles in the adrenegic neurons in this nucleus following monamine oxidase inhibition.

Administration of an antiserum to a nerve growth factor to rats and mice resulted in the

absence of granular vesicles in nerves of the iris and submaxillary gland, but persistence at normal levels of granular vesicles in the vas deferens. This finding agrees with the relative uptakes of d-l-norepinephrine-7H³ in these organs. Earlier hopes that the types of vesicles that cluster principally on the preterminal side of synaptic junctions might help to identify individual nerve fiber terminals on the basis of the neurohumor that characterized it have been partially realized. Proportions of granular and agranular vesicles may vary considerably in different locations and in different physiologic states. It remains to be demonstrated that changes in the population of vesicles provide consistent or sensitive index of physiologic states or of pharmacologic action.

Nerve fibers which terminate on muscle cells function not only to stimulate muscular contraction, but appear also to be necessary for maintaining specific types of metabolic activities in the muscle cells which they innervate. Such "trophic" interactions are under intensive study by the laboratory. Denervation was followed by a 50% decrease in cholinesterase in a period of 3 days. Partial denervation resulted in a loss of cholinesterase activity that was proportional to the fraction of the innervation that was interrupted. Reinnervation following denervation resulted in a slow recovery of cholinesterase activity in the muscle. Cholinesterase activity in hyperneuritized muscle was not greater than that of normally innervated muscle. Denervated muscle exhibits an increased blood flow, a decreased incorporation of C¹⁴-leucine into protein, and an increased uptake of C¹⁴-leucine into the free amino acid pool. Tenotomized muscle does not exhibit this increased uptake in the free amino acid fraction, but does show an equivalent decrease in incorporation into the protein fraction. These and similar studies are permitting us to come to grips at the molecular level with the nature of trophic interactions between nerves and muscle. It is becoming possible to specify which events at the chemical level are sensitive to the presence or absence of nerve, and which chemical events are relatively independent of innervation.

Central Nervous System

In addition to the primary sensory pathways discussed in the section on Sensory Systems, other portions of the central nervous system have received attention. The program of the laboratory is such that over a period of 3 or 4 years most of the major regions of the central nervous system come under active investigation.

This year a detailed analysis of the distribution of the thalamocortical fibers has been undertaken. These fibers distribute to all cortical layers pre- and post-centrally, with heaviest concentration in layers IV and IIIb. In the visual and auditory areas of the cortex, the thalamic fibers distribute heavily in layers IV-IIIa with only occasional terminals in layer I. The increase in our knowledge of the neuronal cytoarchitecture of the cortex paves the way for understanding the manner in which this important layer functions, and provide a rational framework within which to understand problems of dysfunction.

A technical advance made during the past year should be of some help in the exploration of the cytoarchitecture of the brain. It is now possible to apply some neurological staining methods to thin sections cut from specimens of nervous tissue that have been processed in a manner suitable for electron microscopic studies. It should become possible to obtain from the same Golgi stained block one micron thick sections for light microscopic examination as well as ultra-thin sections for study with the electron microscope. This should make possible much more detailed reconstruction of the cytoarchitecture of portions of the nervous system that has hitherto been possible. It has already been possible to show that synaptic spines seen in Golgi stain material correspond to synaptic spines seen with the electron microscope.

Detailed studies have been completed of the normal fine structure of the locus coeruleus, nucleus dorsalis raphe, area ventralis tegmenti, and substantia nigra, with particular attention to the neuronal perikarya of these regions. Studies such as these are extending the information which is available from studies of the gross and light microscopic level to the

level of organization just above the macromolecular level. Only by combining the information available from all of these levels of resolution will it be possible to completely define the structural organization of the nervous system which determines the way in which it marshalls and interrelates incoming information and patterns the outflowing impulses in such a way that appropriate motor responses result.

The closure of the neural folds early in development takes place during a relatively brief period of time. To determine the consequences of a failure of closure of the folds at different levels, closure was experimentally prevented at different levels along the neuraxis, and at different stages during closure of the folds. Among the findings the following are of interest: 1. The neural folds retain the ability to effect successful closure for only a brief period following the time in development at which they approach each other; 2. Failure of closure of the neural tissue at any level leads to overgrowth of the alar plate; 3. Interference with closure of the neural tube at any level greatly increases the frequency of lumbro-sacral spina bifida; 4. Prevention of closure of the neural folds frustrates the accumulation of cerebro-spinal fluid in the ventricular spaces and spinal canal. Under these conditions the optic vesicle which evaginates from the diencephalic wall fails to expand completely, and either makes no contact with the surface ectoderm, or only tenuous contact. If no contact is made, anophthalmia results. If the extent of contact is subnormal, microphthalmia results. Thus, during that critical period in development immediately following the time of closure of the neural folds at which the optic vesicles are expanding, any interference with fold closure, or with the normal accumulation of cerebro-spinal fluid, can result in microphthalmia.

Transfer of Chemical Substances Across the Boundaries of the Brain

Nervous tissue is regionally selective of the chemical changes that occur across its boundaries and between its several compartments. Insight into the types of mechanisms which control the chemical environment of the brain can be gained by comparative studies.

During the past year it was demonstrated that Trypan-blue dye was excluded from the brain of the goldfish as it is from the mammal. As in the mammal, injury to the brain parenchyma permits localized penetration of the dye. The chloride space of the goldfish and rat brain are comparable. However, the thiocyanate space of the goldfish brain is 43%, whereas that of the rat brain is only 10%. By identifying such differences the way is opened for identifying the structural, functional, and chemical bases of such phenomena as the "blood-brain barrier" and the selective compartmentalization of chemical substances by which the brain of each species maintains an appropriate internal environment.

Another approach to this problem has utilized an electron opaque protein (ferritin) to assess the ability of the ependymal lining of the rat brain to take up large molecules. Electron microscopic examination of specimens from 10 to 210 minutes following intraventricular or intracisternal injection of ferritin into rats demonstrated that, although the bulk of the ferritin is taken up pinocytotically at the surface of the ependymal cells, appreciable amounts move between the cells. In another study distribution of ferritin within the brain was followed. The "submicroscopic" pericellular spaces of the brain were found to be diffusion channels and not merely potential spaces. Ferritin crossed the glial border of the cerebellar subarachnoid space and parenchymal capillaries by moving across glial cytoplasm. Once inside the parenchymal interspace, however, molecules are free to reach the plasmalemma of glial and neuronal cells without further crossing glial processes. The identification of pericellular spaces (about 200A wide) as true diffusion channels is a significant contribution to our knowledge of how substances are distributed within the brain. In order to properly understand the dynamics of action of substances ranging from pharmacologic agents to elevated body concentrations of such normal constituents as amino acids, the pathways and kinetics of their distribution in the several compartments of the nervous system must be understood. These basic studies are laying part of the ground work for this type of work.

The inner lining of the nervous system (ependyma) has also been studied with respect to its response to SV₄₀ virus. In the presence of this agent ependymal cells form tumors. Where tumor cells are organized to form rosettes, their interrelationships are similar to the normal ependyma in which apical terminal bars are most commonly composed of a luninal zonula adhaerens followed immediately by a zonula occludens. Where the tumor cells occur in loose array their cytoplasm is highly evaginated, and few or no specialized junctions link the cells. The absence of junctions appear to be associated with those regions which are presumed to be invasive. It is of considerable interest that the presence or absence of cell to cell junctions of the type mentioned above may, at least in this instance, be correlated with a degree of invasiveness of an induced tumor. While it would be premature to generalize this finding, attention will have to be given in future studies to the possibilities which are raised by it.

Degeneration and Regeneration

Attention has been given to regeneration in the central nervous system in lower vertebrates. Regardless of the age in which total or subtotal ablation of the telencephalon is made, no restoration of the ablated parenchyma occurs. After total removal of the telencephalon the site of the lesion becomes covered with a sheet of columnar epithelium. After partial section of a hemisphere, cystic dilations (completely or partially lined with ciliated columnar epithelium) form at the site of the wound. This study has established that even in animals that are capable of regenerating fiber tracts in the spinal cord there are areas of the central nervous system that do not regenerate. The pattern of cellular reaction to central nervous system insults differs in mammals and fish.

Peripheral nerves are commonly isolated from the animal in electrophysiological investigations. A study was conducted to correlate changes in the electrical response of such isolated nerves (cat sciatic nerve, in this instance) to stimulation and to correlate this with the fine structural changes which took place under the conditions of isolation. Unmye-

minated (C) fibers became unresponsive to stimuli after six hours *in vitro*. The myelinated (A) fibers failed after 8½ hours of isolation. Nevertheless, the structure of these fibers appears normal for 12 and 24 hours following isolation respectively. Thus, no correlation could be found between the physiological and ultrastructural changes. Nerve fibers of the central nervous system (spinal cord) degenerate more rapidly than those of the peripheral nervous system when isolated in mineral oil. The electron microscope provides yet another tool for studying the sequence of changes which occurs, the morphology of nervous structures such as peripheral nerve during degeneration.

Regeneration of peripheral nerve has been studied in the sciatic nerve of rats and the vagosympathetic trunk of cats. By removing the nerves to a specially designed recording chamber at known intervals following crushing of the proximal portion of the nerve it was possible to follow, millimeter by millimeter, the progress of an electrically stimulated action potential as it coursed down the nerve. The crushed nerve fibers began to grow very slowly (0.3 to 0.5mm per day) and then accelerated exponentially to achieve a velocity of 3.0mm per day after 18 days. At the level of the lesion conduction velocity reached 80% of normal by 28 days and remained unchanged thereafter. Ten to 15mm distal to the lesion the conduction velocity required 56 days to achieve 80% of normal. Spike height at any point along the nerve was but 25% of normal at 56 days post-operatively. The rate of regeneration in the vagus nerve of the cat is quite comparable to that of the rat sciatic nerve. This is the simplest and most reproducible technique that has yet been devised for studying the rate of regeneration of peripheral nerves. It can be used for pharmacological studies and correlative biochemical investigations. It is planned to utilize this technique for studying the effects of such substances as the drugs of malanonitrile group, colchicine and other agents upon the rate of nerve regeneration. In addition, biochemical studies are planned on the sequence of changes in protein synthesis in spinal ganglion cells during the process of regeneration, with and without treatment with pharmacologic agents that influence nerve regeneration.

Future of the Program

In order to accomplish its mission the laboratory has evolved and maintained a highly diversified folio of projects dealing with the structure and function of the nervous system. While the spectrum of projects under study shifts from year to year in order to meet changing needs, and to take advantage of new opportunities, each of the major components of the nervous system has received attention every year. The laboratory has not committed itself to any one technique or group of techniques, nor to any one conceptual framework. Diversity has been achieved by structuring the staff in such a way that a wide range of approaches are represented in the environment, and at the same time encouraging individual investigators to pursue their problems in depth. The present position and probable future course of our program can best be brought into focus by listing the major areas which are currently under attack.

1. An important goal of the laboratory since its inception is to analyze the structural organization of the nervous system. The auditory, vestibular and visual systems are under intensive gross and microscopic analysis at the present time. The results of these investigations provide a basis for understanding function and dysfunction. The ongoing program concerned with neuronal interconnections among the different levels of the auditory and vestibular systems is a notable example of this type of undertaking. At the present time it is the only major effort in the intramural programs of NIH which deals with the structure and function of the auditory pathways.

2. The laboratory has made increasingly meaningful contributions to our knowledge of the fine structure of the nervous system as revealed by electron microscopy. In the past the cytology of most of the major cell types of the nervous system has been intensively investigated. All but exhaustive inventories of cell organelles in glia, motor neurons, neurosecretory cells, ependyma, etc. have resulted from these studies. More recently the types of junctions that occur between cells (including synapses and myoneuronal junctions) have been analyzed. The structure of the junctions between nerve

fibers and smooth muscle cells has received particular attention. Work on the fine structure of the nervous system will continue to be an important part of our program.

3. Increasing attention has been given to the trophic interactions which occur between parts of the nervous system, and between the nervous system and the periphery. For example, the molecular mechanisms underlying the various types of muscle atrophy (atrophy of denervation, atrophy of disuse, etc.) is being analyzed by studying the control of the protein metabolism of skeletal muscle which is exerted by peripheral nerve fibers. In addition very satisfactory progress has been made in identifying specific interactions among the tissues of the eye and the ear. By developing assay systems which are sensitive to factors arising in one tissue which affect course of differentiation or morphogenesis of adjoining tissues, a way has been opened for the possible isolation of a new class of substances (morphogens). The isolation of such substances would greatly increase our ability to control the regeneration of specific parts of organs. One example of this is the demonstration that there is a water soluble, diffusible influence arising from the neural retina which controls the differentiation of cells in the lens.

4. Intensive studies of the morphogenesis of the eye and the ear are making available detailed flow sheets which describe step by step the differentiation and morphogenesis of these organs. Such flow sheets identify the critical periods during development in which teratogenic influences (pharmacologic agents, genetic aberrations, infections, etc.) will produce specific congenital abnormalities. It has been possible to show that elevated blood sugar is an etiologic factor in the production of congenital lens cataract. Also, interference with the closure of the neural folds during a brief but critical period in the development of the nervous system can lead to microphthalmia. A whole range of auditory and vestibular defects can be predicted on the basis of the specific stage at which an untoward influence is brought to bear. It is anticipated that this work on normal and abnormal morphogenesis will continue. Part of this program will include the use of a

wide range of pharmacological agents in order to assess their effects upon the developing sensory and nervous system, using techniques developed in this laboratory.

5. The laboratory has given attention in the past to the regeneration of the peripheral nerve, especially with respect to neuromuscular specificity. In the immediate past we have developed techniques which make it possible to follow the rate of regeneration in peripheral nerve with some accuracy. It is planned to apply these methods to an assessment of substances and conditions which may favor more speedy and more satisfactory regeneration of peripheral nerves. In addition, attention will be given to the role of protein synthetic systems located in the nerve cell body which may have an important bearing on the rate and quality of regeneration.

6. The development of new techniques, while not a specific objective of our program, is a by-product of the work done in this laboratory. In the past we have contributed importantly to techniques of fixation and staining of material prepared for histologic and electron microscopic examination.

Among other techniques currently being developed is one which may permit routine serial thin sectioning of specimens prepared for electron microscopy.

LABORATORY OF NEUROPATHOLOGY

The Laboratory of Neuropathology, represented by the Section on Experimental Neuropathology, has terminated various investigations which provide new information about the organization of the central nervous system and about the changing reaction of cells along cerebral blood vessels during aging.

It was found that, in the normal brain, there is a fixed relationship between nerve cells and cells of the vascular walls, permitting an interaction. As the consequence of a neurogenic stimulus, mitotic division is induced in a cell along the vascular wall, and a new cell type, the microglia cell, is formed. This cell, because of its location, can be utilized as an alternate transport route between the blood vessel and the neuron. Since the rate of cell division was found to be reduced with increasing age, it was

concluded that the ability to form alternate routes of transport is less in the older than in the younger organism.

A renewed investigation of strands of connective tissue between blood vessels revealed the presence of granular particles, indicating that these strands may serve as intervascular routes of transport. It was also disclosed that cells resembling mast cells are situated near these strands and also near oligodendrocytes. Since mast cells are known to contain many biologically active substances, such as serotonin, which affects the action of oligodendrocytes, these studies open new areas of research whereby the significance of various central nervous system elements under normal and pathologic conditions may be better understood. In order to determine the reaction of various cell types, with special emphasis on the problem of aging, a number of experiments have been performed, such as exercise of both untreated and corticosteroid treated animals, and slight and severe damage of cerebral tissue. Examination of this material is awaiting histologic preparation.

The aim of research projects in the immediate future will be to establish the conditions under which neuropathologic experiments must be carried out and to ascertain the patterns of normal organization, the criteria of functional and pathologic cellular changes, and the factors involved in maintaining normal central nervous function. Thus, some of the current controversial opinions in basic neuropathology may be clarified, and a better understanding of the intricate mechanism concerned with normal functioning of the brain may further our knowledge about the pathogenesis of abnormal functioning of the brain due to temporary or permanent damage. Such projects will eventually entail utilization of the electron microscope and of histochemical techniques, which although pressingly needed, must await availability of other quarters, as planned with transfer to the new research building.

LABORATORY OF NEUROPHYSIOLOGY

Increasing attention has been given in the Spinal Cord Section to the problem of synaptic transmission. This has involved three different aspects of the problem:

(1) The basic mechanisms of synaptic transmission have been studied. A test of the most widely accepted model of chemical transmission at the synapse has been made by measuring cell impedance during the course of synaptic transmission. With a majority of mono-synaptic excitatory synaptic potentials no measurable impedance change occurs although inhibitory synaptic potentials do produce such a change. These findings are consistent with an electrical mechanism of excitatory synaptic transmission and exclude the simplest model of chemical excitatory synaptic transmission. The findings do not rule out chemically mediated synapses located on the cell dendrites.

(2) In view of this result and the increasing attention which is being given in a number of laboratories to the possible functional implications of the structure of dendrites, experiments were started which attempt to demonstrate the manner in which synaptic connections to a cell are distributed to the various dendritic branchings. The question is asked whether the manner in which various inputs to a cell are grouped or segregated affects significantly the way in which the cell integrates these inputs. The results available indicate that there is non-homogeneous dendritic distribution of synapses from different sources and that the distribution can alter the manner in which these synapses interact. Analysis of this data is as yet just begun.

(3) The statistical properties of unitary synaptic events which occur in central neurons is being investigated. The behavior of the miniature synaptic potentials in the absence of stimulation indicates that they are not "random" events in the sense of being independent of one another. This information, coupled with the behavior of the miniature potentials under various conditions of stimulation, will be used to construct theoretical models for the regulation of cell firing.

Interaction between spinal motoneurons which appears to be electrical in nature has been demonstrated. The functional significance of this has not been shown as yet, however.

Evidence has been obtained that some degree of relationship exists between the electrical resistance of a motoneuron, its axonal conduction velocity and its synaptic excitability. This evi-

dence correlates well with experiments of others showing the correlation between the conduction velocity of the axon of a cell and the way that cell participates in reflex activity.

Sensory coding in the auditory system is being examined in the cat by presenting a relatively wide variety of auditory stimuli to the animal and recording single unit responses to these stimuli. Amplitude and frequency modulated stimuli are particularly effective stimuli and appear to offer considerable promise for an analysis of integrative mechanisms in the auditory system.

The ionic basis for the receptor potentials produced by light in the *Limulus* eye is being studied. Preliminary results indicate that chloride ion is not involved in the receptor process and that light may increase the permeability of the receptor cell membrane to sodium.

LABORATORY OF BIOPHYSICS

An understanding of the basic mechanisms involved in the operation of the nervous and neuromuscular systems of animals, including man, is one of the outstanding problems of our time which appears to be susceptible to attack by methods of investigation which are now available.

At the present time the activities of the members of the staff of the Laboratory of Biophysics are mostly directed toward contributing to an understanding of the underlying mechanisms involved in the initiation, propagation and termination of excitation in nerve and similar irritable structures. Without considering the items of growth, maintenance and repair some aspect of the functioning of a sensory receptor of the nervous system, such as the eye, and of the effector cells such as muscles and glands seems to be intimately associated with the effects that electrical potentials and chemical substances have on the ease with which ions cross the membranes of the cells. Scientists in the Laboratory of Biophysics are studying fundamental properties of those natural and artificial systems which lend themselves particularly well to studies of a basic physico-chemical nature. These studies involve the development of sophisticated electronic and mechanical devices, experimental

studies of nerve membranes and artificial systems such as monomolecular films and metal solution interfaces and a considerable amount of thinking about the results and derivation of mathematical models with further studies of their properties.

Progress has been made toward solving some very old problems concerning the basis for the electrical activity in nerve cells. Earlier ideas of ion movement through the cell membrane with the electrical activity resulting from voltage dependent ion permeabilities appear to be essentially correct but only with the recently developed techniques of internal replacement of the contents of the nerve fiber of the squid with known solutions has it been possible to obtain precise information. Calcium has long been thought to be somehow intimately involved in the activity of nerve cell membranes and with the internal replacement of the cytoplasm of the nerve of the giant Chilean squid it has been found that with identical solutions on the inside and the outside of either sodium fluoride or potassium fluoride the nerve membrane shows no rectification or other interesting electrical properties but behaves like a simple negatively charged membrane which discriminates against anions and favors potassium over sodium by about ten to one. Further experiments along this line are in progress and it is expected that the results will provide detailed information about the precise role of calcium ions and interactions between sodium and potassium ions which are required in order to rationally construct a molecular model of the membrane.

Other experimental data has been acquired and analyzed concerning the effects of slowly changing processes. These are very difficult to study but are of importance in connection with repetitive activity, common in sensory systems and in the central nervous system. These studies of slow processes have not yet been undertaken using the combined techniques of voltage clamping and continuous perfusion of the fiber, a procedure which is necessary because of the unknown changes in internal concentrations accompanying applied currents of long duration.

It has been known for almost forty years that there is a significant resting ammonia pro-

duction which increases with activity in nerve fibers and recently we have shown that the ammonium ion can pass through both the sodium and potassium channels to some extent. The Laboratory of Biophysics is not equipped at this time to investigate the possible metabolic relationships but the details of the degree to which ammonium and other ions such as lithium may be able to substitute for sodium or potassium should yield valuable clues as to the underlying molecular structures involved.

Further evidence which strengthens the ideas of a mosaic structure of the membrane has come from the comparison of the dielectric properties of the membranes studied in this laboratory with results of others on artificial thin films of lipid. The capacitance of the natural membrane is greater than the artificial lipid films and varies with frequency. It is suggested that the observed properties of the natural membrane may result from the addition to a basic lipid structure of islands of material having a high dielectric constant and large electrical losses.

Preliminary work has been started on the study of artificial systems and the application of newer physical techniques to natural membranes but this program will not be fully established for some time. Studies of adsorption of a number of cations to monomolecular films of phospholipids extracted from nerve tissue were done in collaboration with a group at the Massachusetts Institute of Technology. Much further work of this type is needed to compare with the emerging results of the studies of the interactions of these ions in the functioning of the nerve membrane.

A considerable amount of theoretical work on excitable membrane properties has been accomplished, much of it in conjunction with the electronic analog computer of the Laboratory of Biophysics and the digital computers at the NIH. Further investigation of the properties of the empirical equations of Hodgkin and Huxley describing movement of ions across membranes have been carried out in connection with the effect of temperature on threshold of excitation. A general statistical model has been developed for the kinetics of ion movements across nerve membranes. This model is based on the concept that the effect of the applied

electric field is a dual one of opening gates or reversing dipoles in the membrane. This determines the percentage of existing channels which are open for the movement of ions which are then driven through by the combined electrical and osmotic forces. Without specifying the chemical nature of the "gates" this model will predict the kinetics of the movements of potassium ions and probably will subsume a number of more specific models which have been proposed with entirely arbitrary assumptions about the molecular nature of the mechanisms involved.

A simplified set of partial differential equations was developed in the Laboratory which retain all of the essential features of the Hodgkin-Huxley equations. These have been programmed for the LINC computer and a film has been made for a theoretical axon demonstrating the phenomena of threshold, propagation of impulses and other physiological phenomena of interest. This is a valuable contribution as a teaching aid and a research tool.

While the study of nerve nets is being given considerable attention by industrial and other laboratories at this time, it is felt that very few problems can be sufficiently well formulated to be accessible to attack by this laboratory with its present resources. However there is underway a highly profitable collaboration with a mathematician in the Computation and Data Processing Branch which is expected to yield a significant mathematical paper concerning the problem of how to interconnect a set of neurons so as to maximize the information storage capacity.

The Chief of the Laboratory has reviewed and extended thirty years of work on excitable membranes and concludes that the simple process of electro-diffusion cannot account for the behavior of the nerve membrane without adding subsidiary processes such as the potential dependent gates discussed above.

There have been a number of cases where individual members of the laboratory have been recognized by invitations to present their views. These have included the Ninth Bowditch Lecture of the American Physiological Society, a Gordon Conference on Biomathematics, lectures at Cornell University Medical College

and Drew University, a work session of the Neurosciences Research Program in Boston, a symposium of the American Institute of Chemical Engineers and a conference in Miami on "Newer properties of perfused axons."

LABORATORY OF NEUROCHEMISTRY

As a result of unanticipated shifts in training schedules, all but one of the Laboratory's complement of research associates departed early in the year. Thus, this has been one of the "quieter" years for the Laboratory. All will be back to normal at the beginning of the fiscal year 1966, when a full complement of research associates, as well as summer "students", report in. The permanent staff was strengthened by the addition of a skilled organic chemist to the Lipid Chemistry Section, where already his contributions to investigations of sphingoglycolipid metabolism are proving most helpful. The long-planned changes in Laboratory office personnel have now been most satisfactorily accomplished. Two staff members have carried out important research assignments abroad in Buenos Aires and Tel Aviv respectively, and active collaboration with numerous intramural and extramural colleagues has continued to characterize all projects of this Laboratory. Specific references to such collaborations will be found in the individual project reports. Here it is appropriate again to acknowledge these mutually beneficial associations and the research progress stemming therefrom and to look forward to fruitful continuations. The Laboratory received the first detailed review of its programs by the NINDB Board of Scientific Counselors since the present organization of the Laboratory four years ago. Their comments and general endorsement were most gratifying.

The general direction of research in the Laboratory of Neurochemistry, as represented by the attached individual project reports, continues unchanged. The major emphasis remains in the so-called basic realm but upon problems with clear, eventual clinical applications. As in previous reports, this emphasis may be most simply summarized as one focussing upon the nature of neural membranes and mechanisms of bioelectrogenesis. In essence what is under

study is interactions upon surfaces and at interfaces between sets of solutions of differing characteristics and metabolic potentialities. The interposition of the interfaces with their resultant surfaces is a direct consequence of membranes surrounding and subdividing the cell. The extremists would on the one hand consider these as no more than fluid-fluid interfaces or on the other as rigid barriers; the truth apparently lying about midway between. For the nervous system membranes are paramount since it is upon and across them that the functional attributes so characteristic of neuronal and neural activity occur. It becomes increasingly obvious that if we are to elucidate the details of such functional mechanisms and if we are to understand the ultimate significance of experimental or clinical dysfunctions, we must know intimately the compositional and structural organizations of these membranes and how these conformations relate to metabolic and physiological events associated therewith.

Few, if any, satisfactory approaches are available or potentially feasible for direct study of any such membranes pure and in isolation. Not only is the nervous system one of the most heterocellular and complex tissues of the body, but it is apparent neurophysiologically that the nerve cell membranes exhibit a complex patchwork of independent but coordinated or ordered events taking place thereon. Thus, what one might consider, if obtainable, a morphologically pure membrane preparation is likely both biochemically and physiologically to be highly "impure", highly complex and heterofunctional. It is for these reasons that the scope of projects and approaches in this Laboratory are so broad and superficially not always so obviously interrelated. And in no sense are the various projects programmed or directed purposively to a single end, but only in retrospect can they be seen to have gravitated by mutual but independent confluence toward a common general goal.

At this stage the various projects are distributed among four or five aspects of the general problem, ranging from studies on "crude" membrane composition and artificial reconstitutions thereof, through investigations of lipid

and protein components of membranes, to evaluation of fluid and electrolyte passage or transport across them and elucidation of the detailed mechanisms involved. The extensive studies by the Section on Lipid Chemistry on the sphingoglycolipids are this year particularly noteworthy. These molecules are essential constituents of various neural membranes (e.g., cerebrosides in myelin sheaths; gangliosides in nerve cell membranes). Current work not only is yielding detailed information about the biosynthesis and turnover of these compounds but also rapidly clarifying the nature of various disorders associated with faulty metabolism of the compounds. Thus, we have seen this year the pin-pointing of the defect in cerebroside metabolism in Gaucher's disease, viz., a lack of glucocerebroside-cleaving enzyme. The techniques developed here are now being applied to the analogous conditions of Niemann-Pick's disease and Tay-Sach's disease. Additional relevance to demyelinating disorders is at once obvious.

Projects in the Sections on Physiology and Metabolism and on Proteins and Amino Acids are closely complementary to the foregoing as the lipoprotein and protein components of membranes continue to be investigated. It is already evident from studies on artificially reconstituted membranes and on preparations of "receptor" molecules that proteins as well as the lipid bilayers are essential to confer upon any membrane-like multimolecular complex the attributes known to be characteristic of natural membranes. Hence, the data from these projects may be expected to provide indications of the types or characteristics of proteins most appropriate in functional terms and to suggest how protein and lipid components are likely to be associated within membrane conformations. Such studies are both highly significant for our further understanding and at the same time most frustrating because of the inherent complexities of membranes and current shortcomings of our investigative techniques.

At the stages of ever increasing complexity are other projects in the Sections on Enzyme Chemistry and on Proteins and Amino Acids, both dealing with cation transport across neural membranes. In the former Section, the na-

ture of the molecular assemblage represented by "transport ATPase" is gradually being unravelled. This continues to be a most significant and fruitful study. In the latter Section, the problems of tissue and cellular compartmentation of cerebral fluids and electrolytes represent the most complex level of investigation short of *in vivo* studies. The recent interpretations suggested by studies on neonatal and developing brain seem to promise extensive clarification of the rather confusing situation in mature cerebral cortex, such that a more meaningful approach to study of cation fluxes in whole cell, incubated slice preparations will not be possible.

In this portion of the annual report for the Laboratory of Neurochemistry, I have repeatedly stressed the complexities involved. This is both deliberate and in no sense an apologetia. What is implied is perhaps best expressed by quoting a recent opinion on this general point by Prof. P. A. Srere, who wrote: "(It has been) said that remarkable scientific achievements have come from 'the methods of simplifying thought by stressing one aspect only of the facts'. None the less, in biological investigations we must deal with systems that are exceptionally complex and we run the danger of choosing model systems that are too simple to advance our understanding. To understand cellular metabolism we must continually search for model systems the complexity of which approaches as closely as possible that of the cell. The models should have been sufficiently examined to enable us to make some simple predictions and explanations of the more complex cellular systems." (*Nature* 205: 770, 1965).

These views are worth bearing in mind when one becomes impatient over rates of progress and when considering the immediate future of on-going projects. The general directions of future developments have been indicated in the foregoing discussion of Laboratory projects and objectives. A specific example emphasizes the viewpoint just cited. We can now reasonably expect that the metabolic derangements in most, if not all, of the lipidoses will shortly be susceptible of detailed explanation. Thus, in the case of Gaucher's disease we may now write in summary form:

- A. (1) Acyl-CoA + serine → sphingosine
Sphingosine + UDP-galactose →
psychosine
Psychosine + acyl-CoA → cerebroside (gal)
- (2) Cerebroside (gal) → ceramide + galactose
- B. (3) Erythrocyte stroma → globoside
(=cerebroside (glu)-gal-gal-N-ac-gal-NH₂)
- (4) a. Globoside → cerebroside (glu)-gal-gal + N-ac-gal-NH₂
b. Cerebroside (glu)-gal-gal → cerebroside (glu) + 2 galactose
c. Cerebroside (glu) → ceramide + glucose

The biosynthesis (A.1) and catabolism (A.2) of galactocerebroside by normal subjects and patients with Gaucher's disease are evidently the same. The degradation of globoside, arising from aging and disposal of erythrocytes (B) also appears to be similar until reaction (4 c.) is reached, where in the Gaucher spleen, the enzyme (*) degrading glucocerebroside is deficient or missing, with consequent accumulation of glucocerebroside. A specific therapeutic approach to Gaucher's disease would thus involve either replacement of the missing enzyme (*) in reaction (4 c.) or inhibition of preceding reactions (4 a or b) to prevent glucocerebroside production. Since we cannot yet "replace" missing enzymes in such circumstances, we are left with the latter alternative, which could conceivably be accomplished. *But* the consequences of such an approach must be clearly appreciated. Erythrocyte degradation and hence globoside production (reaction B.3) is a necessary step in erythrocyte turnover. Blockage of reactions (4 a) or (4 b) would lead to accumulation of globoside or its immediate derivatives and in effect substitute another for Gaucher's disease. In fact Nature has already done the experiment, in which the enzyme for reaction (4 a) is missing, providing us with Fabry's disease. Thus, even though the metabolic complexities associated with the deranged metabolism in the Gaucher spleen have been clarified, the complexities of applying this

knowledge to clinical and therapeutic approaches persist.

One could adopt a pessimistic point of view and admit discouragement at ever really solving such problems. Yet it is precisely the knowledge and appreciation of our areas of ignorance that will in time provide the ultimate answers which we seek. In this sense, the projects of this Laboratory are basically concerned with defining these areas of ignorance and reducing them to manageable proportions. Short of serendipity, this would seem to offer the only reasonable and logical routes for fruitful progress.

LABORATORY OF MOLECULAR BIOLOGY

The research of the laboratory can be roughly divided into two parts, although the overlap between the two has made the studies especially fruitful. A. Structure and Alteration of Nucleic Acids, which includes studies on the evolution of genes. B. Control Mechanisms and Differentiation. Enzymic control mechanisms are the fundament of differentiation, whereas sporulation and germination represent an especially simple example of differentiation which can be studied by a combination of biochemical and genetic methods.

Structure and Alteration of Nucleic Acids

Mutagenic and Inactivating Effects of Hydroxylamine and Its Derivatives

In earlier studies, hydroxylamine had been found to be highly mutagenic with little concomitant inactivating effect when it was used at high concentrations (1 M). At low concentrations, however, it exerted a strong inactivating effect without inducing mutations. Initially, these studies involved phages for which a lethal effect can always be attributed to the alterations of the protein coat. However, when the same lethal effect was observed also for transforming DNA, it became clear that a direct action on DNA was involved. A thorough investigation has now established the following facts. Whereas the mutagenic effect is directly caused by the reaction of hydroxylamine with the cytosine bases of DNA, the inactivating effect comes about indirectly; hydroxylamine, or its derivatives that have a free NOH group, reacts

with oxygen (by means of a metal catalyst) to form a compound which can attack both thymine and guanine in DNA and breaks these bases open. DNA can subsequently not duplicate across the opened base, an effect which is lethal for transformation and may induce large chromosomal alterations in higher organisms. These findings are especially significant since all NOH containing compounds, which have a lipophilic group (enabling them to enter cells), are highly carcinogenic. It is therefore very likely that chromosomal breaks induce cancer. The techniques developed in this investigation make it now possible to rapidly screen many other compounds for their direct and indirect chemical and lethal effect on DNA.

It is intended to determine whether the reaction product of hydroxylamine can directly break the sugar phosphate backbone of DNA or whether the observed breakage occurs indirectly, either chemically or enzymically, as a consequence of the alteration of DNA bases. In order to enlarge the scope of this investigation other chemicals which are also mutagenic, lethal, and carcinogenic will be examined by the above methods.

Studies on the Structure of Chromosomes

The DNA strands which one isolates from cells must be attached lengthwise to one another in chromosomes, because genetic markers are one-dimensionally arranged. It is not known, however, whether or not there are any non-DNA units that link DNA molecules. Such links have been postulated for various reasons which are not decisive, and they have been suggested by experimental results which are not convincing. One argument, for example, has employed the observation that hydroxylamine causes DNA breaks. The authors had not observed any reaction with DNA itself and concluded that some peptide links must be broken. The above finding of a reaction of hydroxylamine with the DNA bases renders this argument invalid.

The decision of whether or not there exist links between DNA is of great importance to the understanding of mutations, chromosome duplication, and especially chromosome function. For it is not known which special properties of a chromosome designate the regions of

DNA that should be used for the synthesis of messenger RNA. The problem of DNA links will be mainly studied by a guest worker who has just joined the laboratory.

*Genetic Analysis of Virus-Induced Mutants of *Escherichia coli**

The properties of virus $\mu 1$, which lysogenizes *E. coli* bacteria and simultaneously mutates them, have been further investigated. The lysogenic state is normally stable because the viral DNA is inserted into the bacterial chromosome. This insertion has been proven by the fact that the genetic distance of markers right and left of the phage attachment site is larger for the lysogenic than for the non-lysogenic state. Wherever the phage is inserted, the corresponding enzyme is absent and the function of the operon interrupted, as has been shown for the β -galactosidase operon which comprises three enzymatic properties. When the virus DNA is incorporated into the β -galactosidase gene the activity of the genes distal to the operator site is also eliminated, apparently because the corresponding messenger RNA can no longer be made. However, when the virus DNA is incorporated into one of the other two genes of the β -galactosidase operon, the function of β -galactosidase is normal and shows the normal induction properties. Recombinants from crosses between lysogenic and non-lysogenic strains often produce strains whose lysogenic state is unstable; in these strains the phage DNA apparently persists as an extra-chromosomal element without initiating virus multiplication.

The Role of Mutation in Evolution

Mutations can be subdivided into different classes according to the mechanisms by which they arise. Since large alterations usually result in rather drastic (often lethal) changes, most mutations that persisted during evolution must have involved the change of a single nucleotide pair of DNA. One can show that mutations of the transition type, i.e., changes $G \leftrightarrow A$ must have predominated, at least in vital proteins. The knowledge of the most frequent chemical changes of DNA suggests further

that the changes from $G \rightarrow A$ should have occurred much more frequently than those in the opposite direction. Since the code between nucleic acids and proteins is now being unraveled it will soon be possible to check from the known sequence of amino acids in protein whether the above picture is correct and whether the same types of mutations have predominated in all organisms.

During the evolution of bacteria at least half of the DNA bases must have mutated once. Throughout this process enzymic properties may have changed and new enzymes may have been formed. In particular, enzymes like dehydrogenases which have a common cofactor, NAD, may have evolved from a common precursor. The examination of three dehydrogenases from *Bacillus subtilis* has indeed shown that they are functionally, immunologically and structurally related to one another.

The enzymes investigated are alanine (Ald), lactic (LDH), and malic (MDH) dehydrogenase. Although their molecular weights differ, their subunits all have a molecular weight of about 35,000, Ald and MDH cross-react immunologically with each other and, although weaker, with LDH. Ald and MDH have at least four peptides in common, two of which occur also in LDH.

Control Mechanisms and Differentiation

Initial Stages of Germination

Germination of *Bacillus* spores is specifically initiated by L-alanine. The spores release dipicolinic acid and calcium, which have formed a protecting coat, and subsequently start the synthesis of polymer molecules. In order to elucidate the mechanism by which alanine initiates germination, mutants of *B. subtilis* have been isolated which were deficient in alanine dehydrogenase. Ald was the only known enzyme that could be involved in germination, since it would convert L-alanine into pyruvic acid and thereby possibly initiate energy production via the Krebs cycle. It was found that Ald deficient mutants germinated at a lower rate but they still responded to alanine. Hence, Ald does support germination, but it is not the only enzyme necessary for this process.

Meanwhile new mutants have been found which do not germinate at all in the presence of L-alanine and germinate slowly (12 hours) in a rich medium. Some of these mutants have an intact A1D but at least some of them miss another enzyme that converts α -aminobutyrate to α -ketobutyrate. Since these mutants can still grow in a glucose-minimal medium, the mutation apparently involves a developmental gene. Further genetic and biochemical studies on the normal and mutant enzyme promise to identify the first step of germination.

It may be worth pointing out that an understanding of germination is not only important as a model case of differentiation, but it is also of interest for two other reasons. First, a detailed knowledge of this process is of importance, for example, for the food industry which has to eliminate Clostridium spores in cans. Second, the process of germination is quite similar to that of fertilization of eggs, which can also be initiated by certain agents instead of sperms (parthenogenesis).

Since various mutations concerned with germination seem to be located in the genetic region of A1D, one may hope to learn, by the same or similar genetic studies, more about the process of A1D induction which was discussed in the previous annual report.

Germination by L-alanine occurs much more rapidly when spores are heated to 70° for 30 minutes before the addition of alanine. This heat treatment can be replaced by exposure of the spores to a saturated solution of calcium-dipicolinate. The Ca-dipicolinate inside can apparently attach to crystals outside the spore and thus liberate certain sites on the spore wall for the action of L-alanine.

Control in Synchronized Bacteria

Bacteria, whose division is synchronized to occur at given times in a mass culture, allow one to examine the development of enzymic processes throughout the mitotic cycle. To accomplish this synchronization a new method has been developed in which B/r bacteria are adsorbed to membrane filters and newly formed buds are continuously eluted. All eluted bacteria are at the same stage of their mitotic cycle and develop synchronously. In these B/r

bacteria DNA, RNA, and induced enzyme (β -galactosidase) synthesis proceeded continuously throughout the cycle. Enzyme induction was possible at any time in the cycle and could be stopped immediately by chloramphenicol. Although these results prove that the mitotic cycle does not influence the inducibility of enzymes, they do not seem to be related to the duplication state of chromosomes. For it is likely that the ring chromosomes of B/r bacteria begin their duplication randomly at any position so that all states of chromosomal duplication are present even in a mitotically synchronized mass culture. Genuine chromosomal synchrony has been obtained by other workers, in different bacteria and in yeast, giving rise to synchronous enzyme synthesis. The induction process of enzymes, however, has not been studied in these cases. Such an analysis should now be possible in germinating bacteria which show excellent synchrony.

Some Properties of Bacterial Cell Walls

During the course of studies on control mechanisms certain observations were made which seemed to be worth following up because they revealed some new properties of bacterial cell walls.

Several enzymes, such as RNAase, protease, and amylase, are known to be released into the medium by *B. subtilis*, whereas in *E. coli* they could be liberated only by lysozyme or, as recently found, by the exposure to the chelating agent EDTA. Most of the other enzymes naturally remained inside the cell under any conditions in both bacteria. It appeared likely that the different behavior of the two bacteria was caused by a difference in the cell wall. In both bacteria the enzyme would move outside the cell membrane, but in *E. coli* the cell wall would be thick enough to retain them in the "periplasm" between cell membrane and wall. One additional enzyme that was found in the periplasm of *E. coli* was alkaline phosphatase. If the general picture was correct the same enzyme should be released (by an exoenzyme) in *B. subtilis*. This was indeed observed. As soon as alkaline phosphatase was formed, after derepression in limiting phosphate, it appeared free in the medium.

When a bacterial culture is aerated, until it reaches the resting phase, the bacteria usually become very resistant to lysis by lysozyme. It has been found that certain bacteria, such as *E. coli*, Clostridia, and *B. subtilis*, lyse immediately when they are exposed, after lysozyme treatment, to high concentrations of magnesium or other cations. Some components of the cell wall are presumably held together by divalent cations and these bonds can be dissolved by an excess of added cations.

Goals

The structure and alteration of chromosomes will be studied in detail. In particular, it will be attempted to obtain definite evidence for or against the existence of links between DNA strands in higher chromosomes. Inactivating DNA alterations, induced by different chemicals, will be analyzed with respect to their types and their induction of pointmutations and large chromosomal alterations. Furthermore, it will be determined which of these chemicals attack DNA directly and which indirectly.

Mechanisms of RNA synthesis and enzyme induction and repression will be studied both in vivo and in vitro. Enzymic changes and other molecular events that occur in sporulation and germination will be analyzed.

LABORATORY OF PERINATAL PHYSIOLOGY

In October 1964, the Laboratory of Perinatal Physiology began functioning under a new directorship. As a consequence, some activities of the Laboratory have been curtailed and other areas of interest are in the process of development. Much of the summary report will be given over to discussion of areas of interests which will characterize the new program of the Laboratory.

One of the most unique and interesting facilities of the Laboratory of Perinatal Physiology, historically and scientifically, has been the free-ranging monkey colonies. The activities of the *Section of Primate Ecology* involved with the field studies have been centered on three small offshore islands, Cayo Santiago, La

Cueva, and Guayacán. Cayo Santiago, at the eastern end of Puerto Rico, was first established as a facility containing free-ranging monkey groups in 1938 with the placing of 409 monkeys on the island for studies of reproductive behavior. In the intervening years and until 1956, the numbers and fortunes of the monkeys on this island waxed and waned. In 1956 when the National Institutes of Health initiated studies of the monkey groups, only 150 monkeys remained. In 1961, the islands of La Cueva and Guayacán, located together at the western end of Puerto Rico, were developed by the Laboratory into a second important field station for comparative studies of reproductive cyclicity and for study of primate behavior. On these three islands has been established a wide-ranging and significant program for studies of population dynamics, reproductive cyclicity, establishment and dissolution of bands, social and reproductive behavior, maternal and child behavior, and many other valuable studies. The singularity of these field facilities derives from the fact that the monkey social groups studied have evolved in an undisturbed natural circumstance for three decades. The resultant social organizations, therefore, are believed to reflect most closely the multitude of factors which direct the evolution of the social phenomenon. The studies which have been carried out in the past and which will continue have been studies of free-ranging social bands and descriptions of behaviors of individuals within these bands. The sizes of the islands are compatible with free movement of the several individual bands which constitute the populations.

Important contributions to our knowledge of the primate situation from the studies of the Section on Primate Ecology include the following:

1. Monkeys in a free-ranging circumstance exhibit an annual reproductive cyclicity with a well defined 3-4 month breeding period in the Fall, and a similar 3-4 month period in early Spring of infant births.
2. The annual reproductive success of adult free-ranging female monkeys approaches 86 percent compared to 43

percent in the caged colony in San Juan.

3. The free-ranging monkey bands exhibit very small to vanishing incidences of abortion and infant loss at the time of parturition in contrast to high incidences in the San Juan caged colonies.
4. Individual social bands on the islands have in a few instances split into two bands. This process is not related to overall band size, but is a reflection rather of complex interactions and relations between more dominant individuals within the bands.
5. The artificial aggregation of animals for extended periods in close confinement is not a sufficient condition for band formation on release. Rather, other factors not yet defined seem to play primary roles in band formation.
6. There exists a remarkable long term stability in the identity and inter-relationships expressed between individuals within social bands.
7. Leadership status in social bands seem frequently related to the social status of the mother. Leaders of bands are found to be male offspring of dominant females.
8. The interindividual regulation and control of behavior within social bands and between individuals of different bands is characterized by aggressive displays and posturing to a vastly greater degree than actual involvement in physical conflict and fighting.
9. Despite the conspicuous aspect of aggressive displays, positive encounters between individuals of a band such as grooming and physical association are quantitatively far more prominent.

In addition to these examples of specific insights into the reality and expression of primate social behavior, there has been a rich flow of normative data descriptive of mother-infant relationships, sibling interactions, male-female behavior during and outside of the breeding season, hierarchical organization within the monkey bands, characterizations of such social acts as grooming, mounting, aggressive dis-

plays, displacement activities, and other manifestations of communication and interaction within the social groups.

The Laboratory of Perinatal Physiology hopes to develop an Experimental Compound Facility in the San Juan area for the experimental analysis of social and reproductive behavior. Experimental approaches represented may be telemetry, endocrinological manipulation by organ extirpation or hormone implantation, manipulation of experiential or psychological variables, and the ablation of brain tracts or brain centers. An assumption underlying many of the anticipated studies is that experimental procedures carried out on the individuals, whether endocrinological, neurosurgical, or experiential should be carried out during infancy rather than during adult life in order to explore the full importance of the factors varied on the development and sustenance of function. Specific brain lesions or other manipulations will, therefore, be carried out in small populations of infant monkeys. Thereafter, these infant populations will be placed in enclosures to develop and express their own specific social organizations. The social orders so evolved will be compared to control groups of monkeys raised under similar circumstance but without experimental manipulation. Such studies should contribute to the solution of some of the most central problems of social behavior and social organization. The enclosure facility will also enable meaningful experimental studies to be initiated and carried out on such problems as maternal behavior, maternal-infant relationship, infant-sibling interactions, male-female relations, communication functions in primate social groups, etc. Within the central laboratory in San Juan, studies will be carried out in several areas of concern as follows:

Physiological Psychology

The main concern will be definition of neural mechanisms underlying perception, memory and learning. Past efforts have been devoted primarily to studies of vision and touch. Future work will continue along the same lines and will be extended to studies also of audition and olfaction. This will allow for cross-compar-

isons between the patterns of organization and functions of the several sensory perceptual systems. The approach emphasized is utilization of formal behavioral training and testing situations which enable the investigator to tie down the perceptual, memory and learning processes, and to express them in terms of learning curves, levels of performance, and in terms of discriminative capacity attainable. The effects upon these functions of various types of brain lesions and of brain stimulations both in cortex and in subcortical mechanisms, can then be defined. Efforts will continue in the direction of defining the capacities of these systems both as intact and impaired mechanisms in the functions of information handling and memory development.

Also included within the area of physiological psychology will be efforts directed toward an understanding of the physiological basis for the perceptual functions utilizing the techniques of modern electrophysiology. Explorations within this context will include investigations of the functional characteristics of neuronal populations and systems which underlie and support perceptual and mnemonic functions. They will include studies of aberrations of normal function resulting from aberrations in development such as occur in the disease amblyopia ex anopsia. They will include also studies of the development of normal function as expressed during intra and early extrauterine existence.

Comparative Neurology

The section on comparative neurology will be concerned with definitions of the patterns of overall organization of the nervous system of the monkey using experimental neuroanatomical techniques. Specific functional systems and neural centers will also be separately studied in an effort to determine the details of intrinsic organization or interconnection within the system. Answers as to the identity and nature of anatomical interrelations between functional systems also will be sought. A comparative approach will also be used to the extent possible to give insight into phylogenetic trends in brain organization. This approach will attempt

to (1) identify and describe patterns of overall brain organization and (2) to specify the anatomical interrelationships of the various mechanisms of the brains of select species of animals within the several classes of vertebrates. It is believed that such comparative studies will give rise to fundamental understanding of structural and functional homologies within the vertebrate series and will supply important clues as to the pattern of organization and the functional import of the brain centers of the nervous system of man himself.

Developmental Neurology

The availability of significant numbers of monkey pregnancies per year places the Laboratory of Perinatal Physiology in a most favorable position for the study of nervous system development. It is hoped, morphological and functional studies may be initiated directed toward furthering our understanding of the relation between structure and function. Particular attention should be paid to the newer techniques which offer the potential of greatly expanding our knowledge of developmental processes.

Experimental Neuropathology

The concerns within the area of experimental neuropathology are threefold. 1) To define the patterns of neuropathological change induced in the nervous system with various types of asphyxial and anoxic insults. These studies will concentrate on oxygen deprivation effects on the fetus *in utero* during gestation and at the time of birth. Studies will also explore anoxic CNS damage in the adult. 2) Definition of the mechanisms through which the various patterns of neuropathological change are induced in the nervous system. These studies will concentrate on alterations in cardiovascular system dynamics testing the assumption that the vascular system represents the weak link in the biological mechanism in response to anoxia. 3) Studies of alterations in the metabolism of single cells of the nervous system under conditions of anoxia. Attempts will be made to relate the alterations in the metabolism and

biochemistry of the cells to the production of reversible and irreversible changes in morphology and function that result from oxygen deprivation.

During the past year the progress of studies in the Laboratory has been impaired by an attenuation of programs due to the hiatus in the directorship of the Laboratory. Many far-reaching decisions concerning development of programs and replacement of professional staff were delayed pending appointment of a new chief of the Laboratory. At the present time the professional vacancies are in the process of being filled and it is hoped that in due course the program will again gain the strength which characterized the Laboratory in the past.

The Section on Neuropsychology has been involved in studies of development of the infant rhesus monkey. Investigations have been carried out to determine whether there is a period of increased impressionability or receptiveness to learning comparable to imprinting in birds. Studies of conditioned avoidance in the infant monkey has revealed no evidence for such a distinct period of impressionability. Rather, following birth there is an initial two or three days of increased responsiveness to sensory stimulation in general followed by more normal levels of responsiveness. In regard to learning, the infant monkey during the first four days does not evidence signs of learning conditioned avoidance. Thereafter, however, learning occurs and the capacity for learning gradually increases into early juvenile existence. Differences are seen between C-sectioned and vaginally delivered infants in that the former exhibit decreased general responsiveness to sensory stimulation during the first five weeks of life. In addition, the C-sectioned animals exhibit depressed capacity for acquisition of conditioned avoidance during the 90 days in which learning has been studied.

A beginning has been made in studying the effects of brain lesions on the patterns of vocalization, general activity, and on the capacity for conditioned avoidance learning in the infant monkey. Lesions in the inferior colliculus and also sham operations prepared with needle penetration through posterior regions of the cerebrum have both resulted in decreased capacity for conditioning at all ages studied up to

90 days. General activity levels and vocalization were not affected by the lesions, but were dependent upon mode of delivery. Beginning studies have been carried out on effects of neocortical commissure section on these functions. Animals with commissure section exhibit lower scores at the onset of conditioned avoidance learning, but end up with significantly better scores than do normals or other animals tested, i.e., conditionability or capacity to learn the conditioned avoidance was enhanced after neocortical commissure transection.

Studies of sleep patterns of infant monkeys have continued in the past year. Sleep in the infant monkeys is characterized by stages similar to those of the adult. Both ages exhibit three overall states of vigilance normally: awareness with normal alert activity, high voltage slow sleep associated with motor quietness, absent eye movements, and active electromyogram, and low voltage fast sleep associated with motor quietness, a low rate of discharge of the electromyogram and rapid eye movements. The amounts of time spent in these different phases is as follows: The percentage of sleep time spent in the low voltage fast phase rises from a low at the time of birth to an intermediate level during the first two days. During the subsequent five days there is a leveling off. Thereafter, a gradual increase is seen through the following twelve months. The overall time spent in any type of sleep is low at the time of birth, increases fairly rapidly to seven days, and, thereafter, again undergoes a gradual decline through the following twelve months.

Studies of sleep deprivation in early infancy, has revealed no post-deprivation alterations in the quantities of time spent in different types of sleep. In juveniles, however, after deprivation of either type of sleep there is a compensatory increase in the proportion of time spent in that type enduring several days after the end of deprivation. For the juvenile, and less so for the infant monkeys, as the period of deprivation was prolonged, there was increasing difficulty interrupting the specific type of sleep being deprived. This was noted in the frequency with which interruption was necessary and also in the current levels required to bring the animal out of the phase of sleep involved.

Animals have been studied with occluders over the eyes placed at the time of birth. Despite visual deprivation, the eye movements during low voltage fast sleep compared to visually normal animals. However, over the first six months of existence, there is a very gradual decline in the amplitude, the time rate of change, and the frequency of occurrence of the eye movements. By six months of age the deprived animals exhibit significant decreases in occurrence of eye movements during low voltage fast sleep compared to normal animals.

Studies of monkeys raised in the wild versus those raised in cages apart from their mothers has indicated that the differences in reproductive behavior between these two groups is less than is generally believed. Both types of animals will accept and care for the infant on vaginal delivery; both groups of males breed normally with evidence for some possible differences in amount of activity, but critical differences occur between the two types of animals in acceptance of offspring delivered by C-section.

In the area of social and reproductive behavior a beginning has been made in assessing normal behavior patterns of the rhesus monkey in the reproductive situation. An understanding is accumulating of the distinctive patterns of behavior of the male and female and of the degree of variation expressed in the caged populations of animals. When proper definition is achieved of these traits of behavior for individual animals, attempts will be made to assess the neural mechanisms underlying and supporting these social and reproductive behavior patterns.

First attempts are underway to explore the neuropathological sequelae of umbilical cord compression during late gestation. It has been possible to reproduce the classical clinical picture of congenital symmetrical spastic diplegia in the rhesus monkey. It is planned to maintain and support the brain damaged infants well into their first or second year of existence in order to characterize the clinical course of their disease and to study the neuropathological changes in the brain under conditions of optimal long-term expression.

A failure of circulation of the fetus has been found in 75 percent of instances at the time of

hysterotomy for umbilical cord compression. This circulatory failure is evident in the poor filling or collapse of the umbilical blood vessels and also in the lack of arterial pulsation in the cord. This results in later abortion of the fetus in 100 percent of cases. The causes for the circulatory failure of the fetus at the time of hysterotomy has yet to be determined. Compromise of the maternal or of uterine circulation has been posited as one potential cause for secondary alteration in cardiovascular function of the fetus. Support for this possibility has not been found in subsequent experiments. Other hypotheses which may account for the fetal circulatory failure are currently under investigation.

In the area of Biochemistry, earlier work has shown that the dissociation curves of whole blood of the monkey fetus lies 6 to 7 mm. Hg. partial pressure of oxygen to the left of the dissociation curve of adult monkey whole blood at pH 7.4. Subsequent studies of the disposition of the dissociation curves of whole blood in monkey infants of different ages yielded wide variations from animal to animal with reference to location of the curves. One suspected basis for the wide variability in location of the curves was a possible polymorphism in adult hemoglobin types. However, studies of hemoglobin types among 200 adults in the caged colony using starch gel electrophoresis has given no evidence for polymorphism. On the basis of the absence of hemoglobin polymorphism in the adult and on the basis of comparison of the dissociation curves of different hemoglobin types with those of their respective whole bloods in man it may be concluded that marked variability of the dissociation curves among the infant monkeys may be related rather to an erythrocyte maturation factor which is as yet poorly understood. Other studies have shown that although at the time of birth fetal hemoglobin predominates in the red cells and in whole blood, by the age of about 55 days the proportion of fetal hemoglobin to adult hemoglobin in the blood has decreased to insignificant levels.

A second area of investigation in the area of Biochemistry has been the nature of the transfer or passage of ketone bodies thru the placenta. *In vitro* studies have shown that NA B-hydroxy butyrate passage through the cho-

riion laeveae obeys Fick's law, i.e., penetrates the placenta by passive diffusion. The rate of diffusion of this charged particle thru chorion corresponds closely to that of urea and is slightly more rapid than that for D-arabinose. There was no evidence for active utilization of Na B-hydroxy butyrate by the chorion laeveae.

A beginning has been made *in vivo* studies of the placental handling or transfer of ketone bodies. Rapid injection of Na B-hydroxy butyrate or of sodium acetoacetate *in vivo* into the maternal femoral vein has revealed that high levels of these compounds can be attained in the blood stream of the maternal animal. Progress of these studies to date have indicated there is rapid inter-conversion between these two compounds *in vivo*. Also, although the conversion of Na B-hydroxy butyrate into Na acetoacetate results in levels in the blood to the ratio of approximately ten to one, in the urine the ratio is approximately the reverse. It appears that the excretion of acetoacetate through the kidneys occurs at a much higher rate or at a much lower threshold than that of Na B-hydroxy butyrate. It is hoped further studies will clarify the question of the *in vivo* transport and permeability of the placenta to ketone bodies.

Several areas of investigation have been explored under the visiting scientists program during the past year. Experimental allergic encephalomyelitis has been produced in six infant monkeys. From six weeks to three months was required for production of clinical symptomatology subsequent to intradermal injection of spinal cord antigen in Freund's adjuvant. There was an acute onset of devastating neurological disease. The animals exhibited varying degrees of hemiparesis or quadriparesis. In two cases the clinical picture suggested a complete brain stem or spinal cord transection. In addition, there was intention tremor, truncal ataxia, strabismus, anisocoria, unilateral and bilateral facial weakness, opisthotonic posture, myoclonus and seizures. Sensory status examinations were not possible. Four of six animals exhibited retinopathic changes consisting of either a diffuse hemorrhagic necrosis of the entire retina or of multiple small hemorrhages and areas of exudate. Four of the six animals progressed to a fatal outcome within two to

four days while two animals survived into a chronic static neurological picture. Neuropathologically there was restriction of gross findings to brainstem and spinal cord in three animals with additional involvement of diencephalon unilaterally in the fourth. Grossly the lesions appear to consist of micro and macro abscesses and areas of greyish infiltration with necrosis. There were multiple small hemorrhagic foci in relation to areas of infiltration and gross infarction. Associated was a variable amount of local and generalized brain swelling. There also was evidence of mild meningeal involvement with localized cloudy infiltrates. Microscopically, the lesions in all four animals exhibited a variable picture of acute and chronic inflammatory infiltration of a perivascular distribution with evidence for microhemorrhages and foci of diffuse diapedesis of red cells. In one case the polymorphonuclear response was particularly prominent and resembled an acute inflammatory response. In the eyes the lesions were comparable in most respects to the lesions found within the nervous system with perivascular inflammatory infiltrate of both acute and chronic types represented. In many foci, particularly in the retina, the process appeared as a periarteritis with intimal proliferation, thrombus formation and recanalization. In almost all cases there were advanced alterations within the optic nerves. These studies will be extended with variations in the type of antigen and adjuvant used and will be carried out on animals of different ages to more precisely define the clinical variation which this interesting experimental condition expresses in the monkey. (See Project No. NIAID-117, Studies on Auto-immune diseases and related immunologic processes, and NINDB-165 O/CH1212C Ocular pathology and experimental allergic encephalomyelitis in young monkeys.)

During 1964 studies were carried out in the Department of Pharmacology at the University of Puerto Rico School of Medicine through a contractual arrangement. This work was devoted to studies of the effects of chronic denervation on the pharmacological and immunological responsiveness of the diaphragmatic muscle of the guinea pig. It was found that denervated muscle becomes sensitive not only to acetylcho-

line, but exhibits a marked and unexpected sensitivity to histamine and bradykinin. Furthermore, strips of denervated diaphragm taken from immunized guinea pigs contract in the presence of small concentrations of homologous antigen. Contractions to antigenic proteins appear to have a genuine immunological origin showing all the features of the Schultz-Dale reaction of visceral muscles, namely: 1) They were observed only in diaphragmatic muscles taken from actively or passively immunized animals, 2) They were elicited only by the homologous antigen or structurally related proteins, 3) Repeated antigen administration caused a desensitization and 4) The dose-response curves were bell-shaped showing a maximum peak instead of reaching a final saturational level.

Intracellular electrical recordings of the activity of denervated diaphragmatic muscle fibers during the anaphylactic reaction showed that the shortening elicited by antigen is a true contraction produced by long lasting volleys of action potentials lasting for more than one hundred seconds. Such long-lasting rhythmic discharges are not due primarily to depolarization, but rather to an electrical instability of the membrane similar to that produced by decrease in the concentration of calcium ions. It was noted that the surface membranes of the denervated muscle fibers became highly sensitive to low frequency, low energy, pressure waves in the surrounding solution; a phenomenon which will be further investigated.

These results are of importance because they show that denervated guinea-pig diaphragm develops new receptors to compounds which are inactive before degeneration of the phrenic nerve, findings which offer an opportunity to study the mechanisms by which the motor nerve controls chemical sensitivity of the muscle membrane. The fact that skeletal muscle is capable of showing anaphylactic responses is technically important, also, since striated muscle fibers are much larger than smooth muscle fibers, and, therefore, more suited for the study of electrical events elicited by antigenic action on sensitized tissues.

Studies have continued on the problem of maternal rubella in the monkey and the transmission of infection to the fetus in utero. Studies of fetal tissues, fetal blood, and amniotic fluid has failed to give evidence for the presence of the rubella virus following or associated with rubella infection of the mother. However, serological studies of the blood of the infant has suggested there may be an immunological response on the part of the fetus to the presence of the virus. In the adult monkey, injection of viable rubella virus produces an active immunological response with antibody formation but fails to give evidence of clinical disease. Offspring of monkey mothers who have sustained virus injection during pregnancy have thus far failed to exhibit developmental abnormalities.

Another visiting group has studied arterial perfusion rates of the nervous system of the monkey fetus and newborn. In earlier studies with adult cats it was shown there exists a remarkable differentiation of various neural structures in terms of arterial perfusion rates. There are distinct differences between gray and white matter in perfusion rates. Most interestingly, various regions of subcortical gray matter and various regions of the cortical surface also exhibit wide differences in perfusion rates. Among subcortical structures in the adult, those showing particularly high perfusion rates are the inferior colliculus and the superior olive. Among the areas of cortex, the primary receptive cortex is characterized by rapid perfusion rates compared to other regions of cortex. According to the preliminary results, the rich differential patterns in perfusion rates seen in the adult animal were not seen in the fetus during gestation nor in the infant during the newborn period. Rather, lesser degrees of differentiation were found between gray and white matter on the one hand and no clear differentiation between various regions of the gray matter wherever studied in subcortical nuclei or in the various areas of cortex. (See Project Report M-CS-OC-(C)-10).

NATIONAL INSTITUTE OF DENTAL RESEARCH

INTRODUCTION

A yardstick of the breadth of purpose in the mission of the Dental Institute is provided by a review of the current fiscal year publication history. With ten months elapsed, a professional staff of 83 investigators has contributed 131 original research papers to 50 different scientific journals, and 23 chapters to 11 books and proceedings. These represented a wide range of science fields in biology, medicine, and dentistry. Although only 10 journals and one proceedings volume could be placed in the dental category, they contained 56 of the 154 published reports and represented principally the products of clinical research programs.

While this annual review of intramural research will be presented from the standpoint of program content rather than organizationally compartmentalized science disciplines, a brief summary of several selected branch and laboratory activities will serve to illustrate the extent of involvement in varied scientific pursuits having relevance to the Institute's categorical responsibility for a specialized health field, as well as the contribution to allied fields of biology and medicine.

It has been emphasized in our **Laboratory of Microbiology** that oral pathoses exemplify particularly well the principle that infectious disease develops only when there is optimum conjunction of microbial, host, and environmental factors. Accordingly, its activity has been quite removed from parochial aspects of oral microbiology, and instead has stressed the determinants of host susceptibility and resistance, and environmental factors such as diet and contact between healthy and diseased individuals. Such a multidisciplinary approach has necessitated numerous collaborations with all other laboratories and branches of NIDR, with other units of NIH, and with outside institutions. It now seems reasonable to expect that the disease-oriented investigations of recent

years, and associated supporting research in gnotobiotics, immunology, virology, microbial physiology, and systemic microbiology, have brought us to the threshold of significant improvement in the control of the principal oral infections.

Multidisciplinary resources available in the **Oral and Pharyngeal Development Section** of the NIDR have made possible an emphasis on basic physiological studies which include (1) elicitation of varied patterns of cry and related arousal response by electrodes placed stereotactically in the brain stem of cat, and the demonstration of action details by correlated pressure recording, sound recording, laryngeal photography and regional cineradiography; and (2) neurophysiological studies of discrete and of slow electrical potential changes in the neuronal networks of the cat brain stem during swallow elicited from the pharynx and during respiration. In addition, there has been continued basic experimentation and clinical application studies of the structure and the motor performance of the mouth and pharynx. Basic anatomical studies have included (1) the vital staining demonstration of differential skeletal growth patterns of the face and cranium of laboratory mammals, (2) the demonstration of detailed development patterns in separate segments of the skeleton cultured *in vitro*, (3) a sequential histological demonstration of responses of bone to deforming forces, and (4) the description in the human of spatial relations between the nasal septum and the base of the cranium during postnatal development.

More recently, efforts have been directed in large measure toward clinical investigations of cleft lip and palate. Unlike the usual research activities of most cleft palate teams and clinics which are related to plastic surgery, orthodontics and speech pathology, the Dental Institute's program has been concerned, in part,

with problems of respiration and feeding during the critical perinatal and postnatal periods, as well as with the preparation of infants for future surgical repair of palatal and lip clefts. Initially, the clinical program developed by the Oral Pharyngeal Section devoted attention to the facilitation of feeding of infants with Robin syndrome (a condition characterized by cleft of the palate, micrognathia and glossoptosis). While previous methods of feeding necessitated mechanical fixation of the mandible, either by intraoral or extraoral supporting devices or by surgical immobilization of the tongue to the anterior oral structures, a new technic was recently developed which utilizes a specially designed device for prone position feeding that prevents pharyngeal obstruction due to glossoptosis, and facilitates active exercise of these structures.

Receiving current attention by the Oral Pharyngeal Development Section is a study designed to analyze the basic mechanisms involved in the maintenance of airway. A pertinent finding, to date, is that such conditions as abrupt reduction in the size of the mandible following bilateral sliding osteotomy, or the procedure of full mouth extraction and denture construction, or the induction of surface anesthesia of the tongue with or without simultaneous anesthesia of the palate, seem to have little effect upon the posture of the tongue-hyoid complex. This observation leads to the speculation that the Pharyngeal Receptor Systems which modify respiration upon stimulation may play active roles in airway maintenance.

In the program of the **Human Genetics Branch**, a considerable range of activity has been pursued; in several instances through collaborative arrangements with other categorical divisions of the National Institutes of Health. These include elucidation of genetic mechanisms controlling the constituents of the various types of saliva; and the study of an hereditary speech defect associated with a previously undescribed neurological spasticity. In the former investigation, it was demonstrated that the concentration of blood group antigenic substances in parotid, sublingual and submaxillary

salivas originated primarily in the mucous portion of the sublingual and submaxillary glands, and that salivary amylase has variable electrophoretic properties in different subjects. The extent to which this variability is a reflection of genetic variability is currently under study. In related investigations of ABH secretor factors and susceptibility to rheumatic fever, no evidence was found for any association between nonsecretor genes and the disease. This is in contrast to earlier reported findings in the literature.

The study of speech impairment was conducted among the Haliwa Indians of North Carolina where all the affected individuals were found to be descendants of an affected male living four generations ago. With particular emphasis on the basis of the triggering mechanism for this defect, other speech pathologists have been made aware of the condition and have recently begun to report its presence among patients in their practices.

Parallel studies in collaboration with the National Institute of Neurological Diseases and Blindness at the Clarke School for the Deaf in Northhampton, Mass., have indicated that there is a clinical thyroid abnormality in about 10 percent of the adult alumni of the school. Clinical chemical studies of the thyroid response to oral perchlorate ion have demonstrated that about half of the congenital deaf have some abnormality of thyroid metabolism, and that more than one metabolic abnormality exists. An hypothesis concerning the effect of thyroid insufficiency during intrauterine development as a cause for deafness forms the basis of this study. In addition to hereditary blocks in thyroid metabolism, other evidence in support of this hypothesis rests on the relationship of hereditary susceptibility to goiter and endemic goiter to the ability or inability to taste phenylthiocarbamide. For example, there is a significant increase in frequency of non-tasters for PTC among the familial deaf but not in the sporadic deaf who have no hereditary history of deafness.

Research animals with an hereditary form of deafness which is a counterpart to the human Waardenburg syndrome have been obtained and are being bred for a study of the

embryogenesis of this ear defect. An associated ocular defect also has been studied in these animals in collaboration with the Eye Research Foundation of Bethesda and the NINDB.

Other studies indicating the diversity of activity and contribution of the Genetics Branch to a broad range of NIH programs include: (1) a study of newborn clinical records from the hospitals of the Division of Indian Health which shows a particularly high frequency of neonatal jaundice among the Sioux Indians of South Dakota, and currently assembled evidence to suggest that this is probably not due to incompatibility in the major blood group systems; (2) a description of the second known family to be reported with a new Rh blood group variant known as e^w; (3) development of a new vacuum system for collecting saliva and a comparative study of the salivary proteins by different methods of analysis; (4) a linkage study between an inherited dyskeratosis and blood groups; (5) a newly described form of hereditary amelogenesis imperfecta giving evidence for the action of X chromosome in females; (6) a description of hypertaurodontism, believed to be the first documented case reported in an American Caucasian; and (7) a study of the relationship of dental caries in the primary dentition to the eruption of the permanent teeth, effects of inbreeding on tooth size, and effects of major physical defects and disease on tooth eruption in children.

In our **Epidemiology and Biometry Branch**, a research during the past year has been directed toward obtaining information to provide a better understanding of the epidemiological characteristics of oral diseases. Continuing collaboration with the Interdepartmental Committee on Nutrition for National Defense extended our studies to Nigeria where, in addition to preliminary gathering of data on the prevalence of dental caries and periodontal disease, the general observation was made that noma (necrotizing stomatitis) occurred with uncommon frequency in children. This lesion results in severe destruction of the oral and facial structures, and without intensive penicillin therapy the fatality rate can reach as high as 95 percent. There was some indication that the occurrence of noma was associated with malnu-

trition and infestation by malaria (*plasmodium falciparum*). Although these and other factors, including measles, tuberculosis, and syphilis, are generally considered to be predisposing, little is known about the specific etiology of noma. While no new evidence of microbial relationships has been forthcoming, the role of fusospirochetal symbiosis, as well as other forms, continues to be suspect.

The Epidemiology Branch has also continued during the current year to participate in the training of dentists who are to act as members of survey teams for projected studies in the six Central American countries. Participation in these surveys will provide data from which to describe the worldwide prevalence of oral diseases and to study possible mechanisms of etiological importance. To date, Dental Institute epidemiologists have participated in surveys in fourteen countries or geographic areas, and oral examinations have been completed on approximately forty thousand persons. In these studies, attention was directed principally toward contrasting findings within and between groups in an effort to better understand the relative significance of contributory factors in the complex etiology of oral diseases. This information continues to corroborate and expand knowledge concerning population characteristics associated with the occurrence of the diseases observed. Work is currently proceeding beyond general epidemiological descriptions to detailed analyses of associations between the occurrence of oral diseases and specific population factors that appear to influence relative susceptibility.

Since the clinical research programs of the Dental Institute generally require a close working relationship between investigator staff and general duty dental officers responsible for patient care services, a transfer of the Dental Department from the Clinical Center to the Institute was effected in July 1964, and reconstituted as a Dental Services Branch. This organizational change did not in any way modify the major objectives or purposes of the dental clinic which have continued to be the provision of optimal dental care for the research beneficiaries of the categorical Institutes. The Dental Services Branch is responsible for providing

complete oral examinations, evaluation, consultation and treatment for patients of the Clinical Center. While it may be an ultimate objective to perform complete dental examinations on all patients, current services are performed only upon request of the attending staff.

In addition to providing patient care responsibilities to other Institutes, the Dental Services Branch renders a detailed, expanded service to all inpatients and outpatients of the National Institute of Dental Research. In this function, it not only furnishes facilities for the clinical investigator staff, but participates in many fruitful collaborative studies. For example, the Branch renders important assistance to the Lukemia Service of the National Cancer Institute in the handling of the myriad of oral problems associated with this disease. In addition, patients with congenital heart defects pose special problems of dental management in both the pre- and post-surgical periods, as do patients with hypertension and rheumatic heart disease. In the latter instance, the absence of proper dental care and preparation, even in such simple procedures as oral prophylaxis, can precipitate a fatal, acute bacterial endocarditis. The same holds true for patients who are to undergo or have undergone cardiac surgery for prosthetic heart valve replacement.

Dental Caries

The demonstration that dental caries in albino hamsters and rats is a transmissible disease requiring oral infection with particular kinds of microorganisms has provided a dynamic base upon which laboratory and clinical research has been building in recent years, and has influenced profoundly the direction of these efforts. First, they have exposed the fallacy in the previously accepted assumption that the cariogenic flora was ubiquitous. Already we know that the failure to develop caries in certain studies, which had been attributed, for example, to dietary factors or genetic resistance of the teeth, was actually due to the absence or insufficiency of a cariogenic flora. Henceforth, all studies must incorporate measures to ensure that the host does, in fact, harbor a cariogenic flora. Second, these findings have encouraged revival of the search for specific cariogenic

bacteria in human caries, which had been quiescent for some 30 years. Already, streptococci, culturally and immunologically resembling those found previously in rodent caries, have been isolated by several laboratories and shown to be cariogenic in hamsters and initially germfree rats.

Up to the present time 36 bacterial isolates have been tested for pathogenicity, but added work will be required before the resulting failures to produce severe or consistently occurring caries can be adequately interpreted. To supplement the animal system presently used, an apparatus has just been designed and built with which bacterial plaques can be grown and sustained on tooth surfaces *in vitro*, by means of automatically cycled media and inocula. Preliminary tests have indicated that this technique may provide a valuable tool for assessing the role of specific microorganisms in caries, as well as methods for inhibition of their metabolic processes.

Since maximum dental caries activity requires an optimum combination of certain bacteria and dietary substrates acting on susceptible tooth structure, an effective means of studying these multiple factors seemed to be by examination of subjects with rampant dental caries. This study has included the use of advanced instrumentation for making intraoral observations and measurements of the caries process and for evaluating local, systemic, and familial factors in children with such advanced type of caries. Concurrently, multifactorial experiments have been carried out in laboratory animals to determine the effects of the strain of the animal, the cariogenic and infectious potentialities of specific types of oral bacteria derived from patients with rampant caries, individual food selection, between-meal-eating habits, and several "inhibitory substances." The fluorescent antibody technique has been used to trace specific microorganisms, making it possible to classify the oral human streptococci in dental plaque and tooth sections. As more knowledge is gained of the parasitic and cariogenic properties of different microorganisms, particularly in tracing specific organisms using the fluorescent antibody method, a more

specific control of potentially cariogenic microorganisms may be developed.

Biochemical research in the area of experimental dental caries continued to explore, as its major effort, the cariostatic effect of phosphates. Additional data pertinent to the possible mechanism of the anticaries action of phosphates support the hypothesis that this cariostatic action is localized within the oral cavity. Thus, it has been demonstrated that sodium phytate, as well as inorganic phosphate, administered in solution by intubation, thereby bypassing the oral cavity, have no inhibitory effect on caries in white rats. Contrary to these findings, comparable groups of rats receiving the same cariogenic diet to which sodium phytate or inorganic phosphate has been added, showed pronounced inhibition of caries.

Localization of the cariostatic effect of phosphate within the oral cavity suggests several specific areas of future research essential to a full understanding of the mechanism of its action. For example, it is conceivable that phosphate additives may influence the spectrum of the oral flora as well as the nature of its metabolism. Whereas dietary factors essential to oral health are most generally placed in the category of a systemic requirement, there is increasing need to evaluate the possibility of inhibiting the cariogenic effect of the diet within the confines of the oral cavity. It is an intriguing concept to consider the likelihood that a food additive in trace quantities might alter the bacterial metabolism to the extent that the byproducts of sugar hydrolysis are not cariogenic. In addition, the mechanism of the phosphate effect may be via the dental plaque and the chemistry of the oral tooth surface.

Other experimental caries studies demonstrated the production of a high incidence of severe caries—both the occlusal and surface type—by feeding a diet containing upwards of 80 percent of commercial sugar in coated corn flakes. As indicated by a normal rate of growth, this diet was adequate in all essential nutrients. However, as in the earlier reported demonstrations of anticariogenic effects of phosphate, this high activity was remarkably reduced by the presence of 0.5 percent or 0.7 percent NaH_2PO_4 in the sugar-coated corn

flakes. These latter experiments are of a particular interest in suggesting the possibility that a sugar treated cereal product may be a suitable food in the human diet to which a phosphate might be added for the purpose of reducing dental caries.

In other laboratory activities it was demonstrated that dental caries in hamsters can be significantly inhibited by topical application of fluoride and/or antibiotics to the teeth using a fitted vinyl prosthetic mouthpiece as a carrier. Most promising results to date in animals have been obtained with topical application of neutral sodium fluoride gels and acidulated phosphate-fluoride gels. A number of non-fluoride compounds also were evaluated, with essentially negative results. These findings encouraged the initiation of a field trial in children to assess the possible caries inhibitory effect of a water soluble gel containing fluoride when applied directly on the tooth surfaces by means of individually fitted plastic mouthpieces. The possible effects of the gel on dental plaque formation and gingivitis also have been under study. Presently, results of three month post-treatment examinations are being compared with findings obtained on initial examinations. Preliminary findings are encouraging and the study, conducted in cooperation with the Department of Preventive Medicine, State University of New York, Buffalo, and the Erie County Health Department, will continue for a full two-year period.

The Institute also has been actively engaged in the study of other aspects of the fluoride-dental caries relationship. This has included continuation of a long-term investigation in which repeated observations of the same children are analyzed to determine the validity of findings from cross-sectional studies of the fluoride-dental caries association. Results, to date, indicate that most assumptions accepted from conventional cross-sectional field studies are valid. For example, children aged 12-14 years, with first fluoride exposure after calcification but prior to eruption of the permanent first molars, showed about the same caries experience in pit and fissure areas as children of the same ages before fluoridation, but caries in smooth surface areas was less by about 31 per-

cent. While detailed analyses of data from this study are still incomplete, the findings are expected to yield a fund of information ranging from actuarial-type tables which will permit estimation of the impact of fluoridation upon public health dental problems for children, to inferences concerning the sequence and mechanics of development and calcification of teeth.

As noted in previous annual reports, studies on the reactions of fluoride with biological mineral have been continuing on a long-range basis. The major aim of these investigations has been to determine by physical methods the effects of fluoride on crystal structure. Accordingly, the earlier utilization of X-ray crystallographic methods for assessing fluoride action on bone mineral is now being applied to the larger crystals of enamel. Results, to date, have shown alterations in diffraction patterns of fluoride treated enamel which suggest changes in crystallite size or strain. Currently, a cold ashing apparatus is planned for construction so that specimen material may be produced that will offer greater promise for obtaining detailed information regarding crystallinity.

With respect to location of the fluoride ion and carbonate in the crystal lattice, recent experiments with infrared spectrophotometry of fluoride treated synthetic apatites are now providing significant data to better define the composition of the inorganic phase of teeth and bones. For example, it would appear that carbonate is located in two general places; i.e., about 10 percent in the hydroxyl positions, and the remainder within the lattice. By such application of infrared methods, it is possible to characterize and assign the infrared absorption bands of pure synthetic apatites, and the band changes caused by the presence of carbonate or fluoride.

Important parallel investigations of the structural changes in carious enamel are being studied by means of microradiography and electron microscopy. Still preliminary results point to the fact that there may be a recrystallization phenomenon occurring at the same time as demineralization takes place. Lending support to this concept have been the evidences in microradiographs of localized high concen-

trations of mineral, and the finding under the electron microscope of atypical crystals in the normally mineral-free prism sheath regions. The complexity of the caries process is thus emphasized, and considerable work with the newer approaches will be required before more understanding can be gained.

Although these studies deal with the etiology of dental caries in all of its complexities, it is hoped that a clarification will evolve of the relative importance of different basic factors concerned in the caries process, with particular reference to the host-parasite relationship as affected by diet.

Experiments with "caries-susceptible" and "caries-resistant" strains of rats indicate that inborn resistance to caries results in part from inability of cariogenic bacteria to maintain residence in sufficient numbers in the respective animals, rather than from genetically determined resistance of their teeth to bacterial attack. Accordingly, such resistance is only relative and can be broken down by sufficient exposure to the cariogenic flora of caries-active susceptible animals, and by appropriate modifications of the diet.

In a study of human dental caries, distinct genetic relationships were found to correlate with the inherited ability to taste phenylthiocarbamide, a trait that occurs in 30 percent of North American white individuals. Findings have indicated that the dental caries experience in the primary teeth of "non-taster" children is 28 to 40 percent higher than in children with the "taster" trait. An extension of this study involves an analysis to test the relationship of salivary thiocyanate level in individuals who have high and low caries rates based on their PTC taste sensitivity. No correlation could be found nor could a relationship be demonstrated between the secretor status for ABO blood groups and dental caries susceptibility. This is in contrast to what has been suggested by other investigators.

The cited findings in laboratory experiments have raised the obvious question of whether parallel processes in caries etiology and pathogenesis exist in man. Accordingly, clinical and epidemiological studies were instituted to relate the applicability of the "specific microbial"

hypothesis to the occurrence of dental caries in children.

In collaboration with a group of investigators at the National Children's Cardiac Hospital in Miami, Florida, a study was initiated to search in human caries for microorganisms similar to those found to cause caries in rats and hamsters. To date, a number of strains of streptococci have been isolated from humans which are serologically and biochemically related to the animal cariogenic strains, and several of these human types have induced caries in both germfree rats and conventional hamsters.

In another preliminary study, young rheumatic fever patients who had received penicillin daily, prior to the eruption of the permanent dentition, were found to have an importantly low dental caries experience than public school children of the same community who had not received antibiotics. This study was conducted in a fluoride deficient area. Presently, this investigation is being extended to include an assessment of the effects of prolonged antibiotic therapy on the occurrence of dental caries in children who are residents of an area with optimally fluoridated waters. Antibiotic levels in saliva also are to be determined and related to selected characteristics of certain oral microorganisms and to the observed levels of dental caries activity.

Under the conditions of the foregoing experiments, dietary sucrose has been found to be practically indispensable for the deposition of precarious dental plaque and initiation of caries, although established lesions progress if the dietary carbohydrate is changed from sucrose to other sugars or to starch. This finding has obvious implications for dietary prevention of caries.

Up to the present time, findings from epidemiological studies indicate that the prevalence of dental caries varies widely; both high and low experiences being observed in the several populations under study. Preliminary analyses have elicited no consistent relations between dental caries prevalence and the respective dietary and nutritional status characteristic of the various samples, beyond the general tendency for low caries experience to be associated with marginal caloric intake and limited use of su-

gar. Thus, within populations, nutritional status in terms of selected vitamins was not found to differ in groups with and without dental caries.

The most recent survey in Nigeria, beginning in Ibadan and extending to about eleven other locations within the country, involved approximately 110 men, women and children, as a sample of the inhabitants, all of whom were examined for physical signs of malnutrition. Blood and urine samples were collected from a random subsample, and data were obtained on dietary intakes and food production. The Nigerian diet is largely a vegetarian one and consists principally of cereals and starchy roots (cassava and yams). It is estimated that these foods account for about 80 percent of the average caloric intake. Preliminary analysis indicates that dental caries in Nigerians is very low compared to findings from reported similar studies in the United States. Interestingly enough, a low dental caries experience in populations subsisting on high total carbohydrate but low sugar diet has been observed during previous ICNND surveys.

Periodontal Disease and Skeletal Disturbance

In striking parallel to the recent advances in our understanding of the pathogenesis of dental caries, there is now substantial evidence that a form of periodontal disease in hamsters can be produced by infection with a hitherto undescribed plaque forming filamentous bacterium and a diet rich in sucrose. The causative bacterium also invades the roots of the teeth and produces a form of caries in the cementum.

Though dental calculus forms in germfree animals fed appropriate diets, it is relatively scant and the structure of its nonmineral phase is amorphous. On the other hand, in conventional animals and man, calculus forms by mineralization of bacterial deposits on the teeth. In other words, although calcifiability is not peculiar to oral bacteria, under normal conditions it is an essential nucleus for calculus formation.

Studies of the sequence of bacterial growth preceding dental calculus formation have necessitated revision of our interpretation of the specific association of filamentous microorganisms with calculus. For example, a wide variety

of oral microorganisms tested in pure culture in a model system *in vivo* have been found to cause calculus deposition equally well, particularly after they die. The specific association of filamentous microorganisms with the later stages of calculus formation, therefore, does not mean that they have a peculiar propensity to calcify. Rather, microbial specificity in this case means that the ecology of the mouth favors their overgrowth on the tooth surface to the exclusion of most other oral bacteria.

In other periodontal investigations in rats and hamsters, it has been shown that calculus fails to develop following total extirpation or duct ligation of the major salivary glands, whereas significant levels form when the parotid gland secretions are left intact.

Allergic inflammation has long been suspected but never demonstrated to be a contributory factor in chronic periodontitis. Experimental support for this concept has now been provided. Repeated deposition of antigen in intact normal gingival pockets of rabbits induces a chronic allergic inflammation at the sites, with characteristic plasma cell infiltration. Furthermore, the treated animals develop homologous antibody in their sera, thus showing that pocket epithelium is permeable to ingress of foreign large molecules. This property is especially significant in relation to possible absorption of the endotoxins known to be formed by the gingival sulcal microbiota. An important contribution to the eventual better understanding of this endotoxin activity is now being provided by electron microscopic investigations of the fine structure of the bacterial cell wall, and its relation to endotoxin formation, and the character of endotoxin itself.

Continuing histochemical studies have yielded new information on the enzymes involved in bone formation and destruction, as well as on the mechanism of action of Vitamin C on bone cells. One of the most exciting results has been the discovery of an enzyme system in cultures of human gingival tissue which breaks down collagen. When exploited, this will undoubtedly lead to a better understanding of the way in which the connective tissue components of the supporting tissues of the teeth are destroyed in the course of periodontal disease.

The influence of viral infection on periodontal tissues has remained a curiosity. When hamsters are inoculated intracerebrally with rat virus, odontogenic tumors develop within both jaws, the first molar roots become greatly thickened by osteocementum, no true periodontal membranes form, the roots of the second molars become greatly shortened, and the third molars, with the exception of those engulfed by odontogenic tumors, show severe alveolar bone loss with exposure of approximately three-fourths of their root lengths.

With the establishment of an *in vivo* method of inducing periodontal disease has come the possibility of evaluating various therapeutic agents. Of those so far tested, striking results have been obtained with certain antibiotic ointments. An additional outgrowth of the foregoing studies has been success in preventing calculus deposition in conventional rats by inclusion of low concentrations of antibiotics in the diet. Such results suggest the prospect of developing effective control procedures in man by local application of carefully selected antimicrobial agents.

The physiological effect of fluoride on skeletal tissues, particularly as a consequence of the use of fluoridated drinking water, has become a subject of increasing interest, stimulated recently by reports in the literature that clinical administration of as much as 60 mg. of fluoride daily causes remission of osteoporosis and Paget's disease. Such therapeutic effects of fluoride on bone tissue have encouraged speculation regarding remission of alveolar bone loss in periodontal disease, as well as possible beneficial effects in other bone dyscrasias, including multiple myeloma. With regard to the latter disease, biopsy specimens of the iliac crest taken from two patients, before and after 6 months of fluoride therapy (20–30 mg/day of fluoride), showed no striking changes in bone chemistry or in X-ray diffraction pattern other than an approximate 2.75 fold increase in fluoride content.

In a clinical periodontal study of alveolar bone loss in children, it was found that in hypophosphatasia, premature exfoliation of primary teeth occurs because of their "shell"

shape structure and the presence of little alveolar bone. The affected teeth show little or no cemental covering.

Generally, periodontal diseases constitute a much greater problem than dental caries in most of the population studies conducted by Institute epidemiologists. Disease levels lower than those commonly observed in groups within the United States were seen only in remote areas of Alaska and in primitive Jivaros of Ecuador. Gingival disease with relatively little tooth loss from this cause was reported from Ethiopia. Elsewhere, the onset of periodontal diseases was early and advanced destruction was common even in young individuals. Extremely high levels of disease were observed in Lebanon, in Trinidad, and in the three countries of southeast Asia—Viet Nam, Thailand and Burma. Despite a favorable dental caries experience, tooth loss resulting from destruction of the periodontal tissues in some of these populations was high.

Local irritants such as deposits of oral debris and calculus appear to contribute significantly to tissue changes characteristic of periodontal disease. Relationships between these local irritants and the severity of periodontal diseases have been observed consistently in all populations examined. The combined effects of oral debris and calculus are sufficient to account, statistically, for over half of the observed variance in the clinical severity of periodontal disease as determined by the Periodontal Index.

Given equivalent levels of oral cleanliness, the severity of periodontal disease also increases progressively with advancing age. Whether age represents a longer period of time for the deleterious action of local irritants, or whether there is some attenuation in the resistance of the periodontal tissues to local irritation with advancing age is not yet determined.

Further analyses have been undertaken during the year to explore the possible linkage between nutritional status and the occurrence of periodontal diseases. Results generally supported findings from previous studies; i.e., there was no appreciable relationship between periodontal health in a population and its nutritional status as determined from selected

biochemical assessments, including serum ascorbic acid and Vitamin A and urinary thiamine, riboflavin and niacin.

Calcification and Normal and Abnormal Growth and Development

New information on mechanisms by which cells grow and organic matrices become mineralized is being gained from tissues other than bones and teeth. The purpose of such studies is to investigate regulating factors in growth and calcification in systems which for one reason or another are less complicated biologically, more amenable to the technical procedures available, or convenient for actual experimentation.

Basic studies on the regulation of cell growth at the molecular level have shown that the alteration of a population of nongrowing human lymphocytes to a state of rapid growth is closely associated with marked and characteristic changes in the pattern of RNA metabolism. The cells were stimulated to grow by addition of phytohemagglutinin (an extract of the kidney bean), and metabolic changes studied during the course of cell enlargement and division. To date, it has been found that stimulation by phytohemagglutinin produces an abrupt breakdown of a significant proportion of the preexistent cellular RNA, with almost immediate onset of accelerated synthesis of new RNA. The earliest type of RNA produced in this accelerated fashion appears to be non-ribosomal RNA. After a lag of one to six hours, ribosomal RNA synthesis is accelerated. Over the course of 72 hours in culture, up to the time of mitosis, the rate of synthesis of both ribosomal and non-ribosomal RNA increases. However, the synthesis of a labile, non-ribosomal RNA predominates, and after 72 hours, nearly 60 percent of the rapidly synthesized RNA appears to be of the labile type.

In other fundamental research approaches, the calcified tubes formed by marine worms have afforded the opportunity to study crystal nucleation, growth and distribution under the influence of varying environmental factors. Thus, calcareous corpuscles in tapeworms have permitted investigations to be made with phosphate complexes which are originally amor-

phous and can be made to crystallize experimentally; and artificially calcified mitochondrial isolates have yielded new information on chemical factors that influence mineralization. Parallel studies of arteriosclerotic calcifications also have provided the investigator with a unique mineralizing system in which apatite is laid down, but in a matrix different from that of bone or tooth.

In an extension of the above cited projects, the mechanism of calcification has been studied, *in vitro*, by the use of aortic or nuchal ligament elastin as an organic matrix. Findings from this investigation indicate that a minimal level of calcium and the presence of free sulphhydryl groups is required in the matrix for calcification to take place. Calcification also was found to occur *in vitro* under the same conditions without matrix if a sulphhydryl compound is added in solution. These results suggest at least one way in which protein matrices contribute essential elements to the calcification process.

In crystallographic studies of phosphates, a series of calcium phosphates made synthetically have been examined by X-ray diffraction, infrared spectrophotometry, and electron microscopy and diffraction. Among the problems investigated have been the lattice parameters of pure hydroxyapatite, the possibility of octacalcium phosphate formation in biological calcification, and the location of the carbonate ion with respect to the apatite structure. These are all long-range projects, and the data have an important bearing on the as yet incompletely determined atomic structure of the biological apatites, as well as their chemical reactivity.

In previous years, Dental Institute studies have been concerned largely with the embryology of enamel and dentine, and have dealt with matrix elaboration and calcification. This year a principal effort has been directed toward investigation of an earlier period in tooth formation, when differentiation of the cells of the primordial dental papilla into secretory cells destined to form dentin takes place. The events which occur at this stage are complex, and it is of some importance to develop an understanding of the factors controlling differentiation as well as an insight into the interdependence of

one tissue type on another in order for the event to occur normally. It is often this far back in the process of tooth formation that pathological disturbances are determined. In the present study, utilizing rat and rabbit material, an *in vitro* culture system was developed, which gave interesting information on the nutritional needs of the mesodermal dental papilla in order for odontoblasts to form. In addition, it was found that unless enamel forming cells, actually derived from ectoderm, are in close contact with the papilla, formation of a functional odontoblastic layer does not occur. At this point a technique is at hand by which a variety of influential factors can be studied experimentally under quite well defined conditions.

In another set of experiments with developing teeth of rodents, it was found by histochemical methods that essentially all of the enzymatic activities associated with matrix secretion and calcification begin at a time subsequent to the differentiation of the enamel and dentin forming cells.

For many years considerable emphasis in our Institute programs has been placed upon contributing to the solution of the problems of defining the structural organization of enamel in prismatic units. As the concepts of general structure have become clearer the tendency has been to move in the direction of investigations of crystal structure in fine detail. This year a new test object has been found in shark enamel, where hydroxyapatite crystals are extremely symmetrical. Inasmuch as shark jaws contain teeth with enamel in many stages of development, it has been possible to observe crystal formation in a series of developmental stages, and thus to complement the information previously gained from studies with rodent material.

In connection with these and other studies of calcified tissues, improvement in microradiographic instrumentation have been affected, including construction of an interchangeable target X-ray tube, with which radiation of several different wavelengths can be generated. With this selection of X-rays it is possible to produce microradiographs with more adequate contrast

and to impart more significance to the absorption images.

With more adequate information at hand about normal enamel, it is becoming possible to begin work on abnormal development. There are various ways in which defective enamel can be produced experimentally, and the toxic factor employed in the present studies was tetracycline. This drug has proved useful in that reproducible pathosis is produced and because the actual localization of tetracycline in the tissue can be detected by its fluorescence under ultraviolet light. The response of developing enamel in the rodent has been studied microradiographically and electron microscopically. Depending on dosage with tetracycline, hypoplastic defects have been created which range from alterations in number and size of inorganic crystals to loss of prismatic structure and extreme aberrations in mineralization. This experimental model promises to yield considerable data on enamel mineralization in general.

A similar study pertaining to calcified tissue has had as its objective an evaluation of the effect of tetracycline on bone. Reports in the literature indicate that tetracycline interferes with the growth of long bones in infants and adversely affects mineralization in a wide range of animal species. Recently, an examination of the effect of tetracycline on calcium metabolism of bone in white rats disclosed that maternal and fetal radiocalcium uptake is increased as the dietary concentration of tetracycline increases. However, tetracycline ingested at levels in excess of those employed therapeutically in man does not affect fetal growth or produce overt developmental anomalies. Placental transfer of calcium, likewise, is not altered selectively by tetracycline administration. It should be emphasized that the tetracycline in these experiments was administered intragastrically. Other reports in the literature indicate that rats receiving tetracycline by the oral route retain extremely little of the dose in their bones after one week, whereas intraperitoneal administration is followed by significantly higher levels of retention.

A survey of children in a pediatric practice, in an urban community and in a rural area,

was conducted to discover the prevalence of tetracycline induced defects in the dentition. Twenty-six percent of the 146 children in the first group, 5.8 percent of 292 children in the general urban population group, and 3.4 percent of the 147 children in the rural population group were found to have stained or hypoplastic enamel which fluoresced when viewed under an ultraviolet light. Correlations between fluorescence and ingestion of tetracycline during the period of tooth formation was high (92 percent). From these data it is assumed that administration of tetracycline is among the more common causes of non-carious tooth defects among children today.

Another clinical study of congenital malformations (in collaboration with the Perinatal Research Program of the National Institute of Neurological Diseases and Blindness) related to socioeconomic, medical, and genetic factors has included observations on cleft palate and lip, anencephaly, polydactyly, syndactyly, spina bifida, meningocele, congenital heart disease and hypospadias. Subjects for study were selected from live and stillbirths registered to date in all institutions participating in the collaborative project on cerebral palsy, mental retardation and other neurological and sensory disorders of infancy and childhood. A normal control group also has been included. Preliminary tabulation of approximately 20,000 live births has shown the total frequency of these major congenital malformations to be 1.7 percent. In addition, 62 cases of stillbirths were found which had at least one of the major congenital defects.

A study of primitive Indian populations in Brazil showed a striking absence of malocclusion, there being only five percent with a significant degree of dental malalignment. This contrasted strikingly with an adjacent Indian tribe which had extensive contact with white civilization for 20 to 30 years and which showed the frequency of malocclusion to be 52 percent. Fourteen percent of the latter group were classed as having severe abnormalities of occlusion.

The effects of inbreeding on dental characteristics and congenital malformation have been assessed and reported in publications

based on a study done on offspring of consanguineous marriages in Hiroshima and Nagasaki, Japan. This investigation showed that recessive genes probably play a significant role in the development of malocclusion, although environmental factors are of significance. In general, however, it would appear that genetic factors are more of a predisposing nature than causative.

Analogous studies of the facial and cranial skeleton have continued in subjects distorted by anomalies and/or neurological impairments. These have included standard methods of radiological cephalometry and also adaptations of laminography which demonstrate the mid-line structures. In clinical application, evaluations of the basic orthodontic methods of tooth displacement have been made, using precision techniques to portray spatial changes of teeth.

Epidemiological investigations were continued during the year to provide additional data concerning the relation between certain dental arch dimensions, malalignment of the teeth and other orthodontic problems. Utilizing methods developed during the preliminary phases of study, results now indicate that malalignment of the dentition is inversely related to dental arch width, but is relatively independent of tooth morphology and arch length. Further analyses of these data are still in progress.

Analysis of clefts of the face, lip and palate occurring among the Haliwa Indians of North Carolina indicates a prevalence 6 to 12 times that of the North American Caucasian population. Kindred data show that 28 cases so far described are all the descendants of one woman born seven generations ago. If genetic factors are involved in this defect they are not simple genetic traits. A new method of radiographic examination for possible carriers of cleft lip and palate is being developed in this study and in a study of 200 families at the Lancaster Cleft Palate Clinic, Lancaster, Pennsylvania.

Reports of cleft palate among Indians born in Public Health Service Hospitals in the state of Montana tend to substantiate a previous report of an extremely high prevalence rate in this area (1 in 276 births). Factors such as the degree of inbreeding, the degree of Indian blood, parental age, parity, season of birth,

geography, certain socioeconomic factors and cultural variables are being analyzed for clues to the etiology of this condition. A permanent birth record collection system is now well established, and methods of analysis have been developed for these data.

Studies of upper respiratory and feeding functions have continued in an increasing number and variety of subjects impaired by anomaly, such as the Pierre Robin Syndrome. Utilizing methods of cineradiography, cinephotography, respiratory displacement, and sound recording and spectrographic display, a considerable body of data has now been assembled on selected patients having cleft palate and other oral and pharyngeal anomalies. By these technics, plus transducer methods of observation of tongue contacts and margin pressures made in parallel with recordings of speech and swallow, there has come about a redefinition of disabilities in terms of deficiencies and compensations of pharyngeal actions, rather than the overt deficiency of palate and related structures. These demonstration methods have also been extended to subjects having neurological impairments of the oral and pharyngeal area, and initial descriptions have been made of the particular distortions of feeding, of pharyngeal airway maintenance, and of vocalization in subjects having spastic dyskinesia, athetosis, lower neuron disorders, and regional kinesthetic sensory disorders.

In another area of investigation, a number of chemically related antihistaminic drugs (meclizine, chlorcyclizine, cyclizine) have been demonstrated to induce specific oral-facial malformations in the Sprague-Dawley rat. Other antihistamines (diphenylpyraline, methapyridine, chlorphenoxamine, diphenhydramine, promethazine) do not induce malformations even when administered at toxic levels at critical stages of development. This indicates that the drugs induce the malformations via the metabolism of their own chemical moiety, rather than by a common antihistaminic pathway.

Preliminary metabolic studies also have demonstrated that a demethylated compound (nordchlorcyclizine) is distributed throughout the maternal and fetal organisms of animals treated with meclizine or chlorcyclizine. Other

possible breakdown products of chlorcyclizine; i.e., p-hydroxybenzophenone, p-chlorobenzophenone, p-chloro-p'-methoxybenzohydrol, 4 chloro-4'-hydroxybenzophenone, and norchlorcyclizine, were tested for their teratogenic potential but only northorchlorcyclizine was found to induce congenital malformations. It is significant that these abnormalities are exactly the same in character as those induced by meclizine and chlorcyclizine.

In order to evaluate any possible differences between animal experimental results and human experience, studies on the metabolism of chlorcyclizine have been carried out in a number of mammalian species, ranging from the opossum to man. In all instances chlorcyclizine has been found to be metabolized to the demethylated compound which readily crosses the placenta of the rat, the Duroc swine and the Rhesus monkey, and is found in comparatively large amounts in the fetus. Three hours after administration of chlorcyclizine to pregnant swine, the compound is metabolized to the nor-compound. In the monkey the rate of metabolism is slower and requires 24 hours for a complete change. Preliminary studies in the human indicate that chlorcyclizine is metabolized to the nor-compound.

As is the case with other drugs (i.e., phenobarbital), chronic treatment with chlorcyclizine does not yield the same results as acute treatment. Chronic treatment leads to a diminution of the amount of the nor-compound found in the maternal and fetal organism as compared with the amount found under acute treatment, and there is a corresponding lowering of the incidence of malformed fetuses. It should be noted that "acute" treatment is determined as the minimal time (4 days) required for the drug to induce its teratogenic effect.

At the present time we are able to induce with ease, cleft palate in different species of animals by different teratogenic agents. Because of this we are now in a position to study the comparative pathogenesis and histochemistry of drug-induced oral-facial malformations. This currently involves the assessment of the distribution and metabolism of individual connective tissue mucopolysaccharides in the

palatine shelves and their relation to normal palatal closure and to inhibition of closure with chlorcyclizine and hypervitaminosis A.

Proteins and Enzymes

The mechanism of action, specificity, and utilization of proteolytic enzymes in the experimental modification of protein and enzyme molecules contributes to a major extent to the resolution of molecular structure. Knowledge of specific functions and interrelationships of enzymes, obviously applies directly to an understanding of all biochemical processes.

Major procedures in this research pertain to the proteolytic enzymes: carboxypeptidase A and B, chymotrypsinogen C, aminopeptidase A, and liver transglutaminase. Having perfected a sensitive procedure, kinetic constants were obtained for carboxypeptidase A and B, and for a number of their metallic derivatives. Studies involving ester substrates have furnished strong evidence that no amino or iminazol group in the enzymes participates in ester substrate binding. Carboxyl groups may be important in these catalyses.

Continuing studies of the mechanism of procarboxypeptidase A activation show that chymotrypsin alone activates this zymogen. It would appear that this information may become the basis for a new concept of the mechanism of biological regulation and the sequential events which occur during protein digestion.

The activity of aminopeptidase A in blood serum was studied with the result that this enzyme inactivated angiotensin by hydrolyzing an N-terminal aspartyl peptide bond. As a result of this discovery it has been postulated that this enzyme is instrumental in controlling angiotensin levels in blood, an observation of obvious interest in problems of hypertension.

Collagen is the major structural protein of vertebrates and many invertebrates. The protein, initially synthesized in the form of monomers, undergoes aggregation outside the cell into fibers of high mechanical strength. The stability of the fibers is further enhanced by the introduction of covalent crosslinks. The manner in which collagen performs this function is being approached through the study of

the structure of the collagen monomer. Thus, it has been shown by denaturation of the molecule and chromatographic fractionation of the products, that each collagen molecule is initially formed from three chains (the so-called α chains) which are not identical but which have the same molecular weight; i.e., about 100,000. In the case of a collagen from codfish skin the chains have been resolved chromatographically. With other collagens it has been possible to resolve only one chain, the α_2 chain, from the other two, the α_1 fraction.

Further details of the structure of collagen were obtained by employing a procedure for the specific cleavage of polypeptide chains at methionine residues. Since each α chain from mammalian collagens contains about eight methionine residues, about nine peptides should be released. The α_2 chain gave the expected number while the α_1 fraction gave twice the expected number. This indicated that the α_1 fraction must contain two kinds of chains, designated α_1 and α_3 , and demonstrates the nonidentity of all three α chains in mammalian collagens, as shown chromatographically for codfish.

The use of a collagenolytic enzyme from tadpole has given additional information on collagen structure. Characterization of the products of the action of this highly specific enzyme on native collagen shows that all three α chains extend the full length of the molecule rather than having a folded configuration.

Elastin is another major structural protein in tissues whose function requires elasticity. The property of elasticity is achieved by a randomly folded, but crosslinked, chain structure. It was further shown by isotope studies that the amino acid, which is a crosslink, is synthesized from the side chains of lysine residues in peptide linkage. This process apparently proceeds through an as yet unidentified intermediate which then condenses after an elastin precursor is deposited extracellularly. Either copper deficiency or the presence of a lathrogen inhibits the formation of crosslinks.

According to a current hypothesis, the initial stages of protein synthesis involve the soluble ribonucleic acids as the specific carriers of activated amino acids. Soluble ribonucleic acids

differ from other types of nucleic acids in that they contain small amounts of methylated bases, in addition to the usual bases. The biochemistry of these methyl groups in genetic coding systems is a fundamental objective of these studies. In studies designed to elucidate the role of methylated components of soluble RNA, the crucial factor has been the availability of an unusual mutant of *E. coli*, which under a special set of circumstances has the capacity to produce soluble RNA, without methylated components.

Studies thus far have shown no requirement for methylated bases in the amino acid acceptor function of RNA, when measured under classic *in vitro* conditions. Such findings have led to the suggestion that methylated bases are not involved in the amino acid acceptor activity of soluble RNA. However, as a result of other studies, it has now been shown that a quite sensitive probe for evaluating the function of methylated bases in RNA resides in the use of cross-reacting amino-acid activating enzymes. Thus, advantage has been taken of the fact that the leucine activating enzyme from yeast has the capacity to attach leucine, not only to soluble RNA from yeast, but also to soluble RNA from *E. coli*. In such a system it has been demonstrated that while soluble RNA from *E. coli* containing methylated bases can be acylated with leucine by the enzyme from yeast, the soluble RNA devoid of methylated bases has lost this activity. It was concluded, therefore, as a result of these procedures, that methylated bases must be implicated in the amino acid acceptor activity of soluble RNA. Furthermore, recent studies have compared the activity of normally methylated and methyl-deficient soluble RNA in a variety of systems, all of which have shown identical activity. In addition, it has become evident that different levels of methylated bases are associated with the RNA molecules specific for different amino acids.

The physical separation of soluble RNA containing methylated bases from soluble RNA deficient in methylated bases has been accomplished by using column chromatography on methylated albumin coated Kieselguhr. This technique makes possible, for the first time, the preparation of soluble RNA free of methylated

bases, and allows more critical studies of such macromolecules. Thus, it has been possible to show unequivocally that unmethylated RNA is formed by *de novo* synthesis in the absence of a methyl donor and not by removal of methyl groups from pre-existing RNA.

Work with a DNA-ribosome complex, done in collaboration with investigators from other laboratories, has produced electron micrographic evidence of the postulated linkage of DNA with ribosomes by RNA strands.

Oral Soft Tissue Lesions and Clinical Diagnosis and Treatment

Clinical dental studies concerned with oral diseases involve all the tissues of the mouth—the teeth, the supporting bone, the periodontium, the tongue and the mucous membranes. Rampant caries, periodontitis, periodontosis, pulpitis, the stomatitides—aphthous, viral and desquamative—congenital anomalies and malocclusion are conditions receiving the most attention in our program. However, since it is clearly recognized that one cannot separate the state of the oral structures from the health of the entire body, clinical investigations necessarily include related laboratory studies on physiological and biochemical mechanisms, as well as studies of similar disease conditions in model animal systems.

In more specific terms, examples can be cited of projects designed to (1) evaluate the effects of post extraction bone resorption related to periodontal involvement; (2) analyze parotid saliva collected from slight and heavy calculus formers; (3) study the relation of blood types to the incidence of post-surgical sequelae in third molar extractions; (4) understand the significance of "cementum granules" on periodontally involved teeth; (5) develop clinical methods of control of rampant caries in the young adult; and (6) evaluate various restorative procedures on human teeth.

In an attempt to find more ideal restorative materials, collaborative research is being conducted with the Eastman Dental Dispensary, and with an industrial (non-dental) manufacturer to develop and test adhesive filling materials that will mechanically and chemically bond with tooth structure. The biologic effects on the

human dental pulp tissues of two such experimental materials are currently under investigation. However, because the Clinical Center can supply only about 30 human teeth per year for such studies, it has been necessary to supplement our needs with contract arrangements with several universities and other government facilities.

Studies of the human dental pulp have continued to emphasize the description of changes induced by dental drilling procedures and by various restorative and related materials, such as cavity liners. These investigations have furnished the dental profession with some very practical information on operative procedures, particularly in regard to optimal cutting speeds, the proper use of coolants, and modifications in technic necessary for the safe placement of amalgam.

Because of the delayed and reduced inflammatory response of the pulp following high speed cutting technics, the time lag period for the production of reparative dentin has been greatly prolonged. Thus, dentinal tubules remain open and permit the toxic or irritating products of sterilizing agents, cements, and silicates to permeate to the pulp tissue and cause further damage. This slow response of the pulp to repair is creating a formidable problem in restorative dentistry, especially in the field of full mouth rehabilitation where often the entire coronal dentin is exposed. Experimental drugs designed to reduce sensitivity of teeth (i.e., corticosteroid compounds) and to more effectively seal the dentinal tubules are being sought, as well as drugs and technics to reduce the lag period in which reparative dentin is formed. In a recent study, experimentally prepared cavities in teeth of humans (scheduled for routine extraction in connection with orthodontic or periodontal treatment procedures) were washed with a steroid formula containing 1 percent prednisolone in a vehicle of parachlorophenol, metacresyl acetate, and gum camphor before restoration with zinc oxide and eugenol. Extraction of the teeth was accomplished at varying time intervals following experimental treatment, and the specimens then prepared for histological examination.

The results were significant in that the inflammatory response to cavity preparation was minimized about 50 percent when compared with a control group of teeth that had received no medication prior to restoration. On the other hand, the fact that reparative dentin was found at approximately the same time interval and occurred at a similar rate in both groups indicates that there was no enhancement or interference with the healing process.

In ancillary laboratory studies in which the tooth pulps of germfree rats were surgically exposed, it was apparent that infection rather than trauma is the critical determinant of damage. For example, though the experimental cavities became impacted with food and other debris, pulpal reaction was minimal in the germfree animal and eventually the exposed area became closed over by secondary dentin. In contrast, the exposed pulps of conventional, germ-bearing rats showed extensive necrosis with no evidence of healing.

Continuing investigations in the past fiscal year with herpes simplex virus have increased our understanding of the phenomena underlying recurrent fever blisters (cold sores) caused by this virus. Using a refined serological technique, it was shown that the virus strains isolated from successive recurrences in the same person are often serologically distinct; that is, partially resistant to the person's pre-existing herpes antibody. Also, primary oral ulcerations have been induced with herpes virus in the oral mucosa of rabbits with hitherto undemonstrated infection of mucous gland ducts. Surprisingly, this virus has been isolated repeatedly from the saliva of rabbits many months after intra-abdominal injection, i.e., without oral infection or other overt lesion. Finally, a model system of persistent herpes virus infection has been established for the first time without homologous antibody in tissue culture, exhibiting alternating periods of latency and cell destruction with virus production. This parallel to the course of events in spontaneous human herpes virus infection should help define the mechanism of viral induction out of latency.

Parallel studies of recurrent aphthous ulcerations (canker sores), a common debilitating and painful oral disease of unknown etiology,

also have progressed with notable success during the year. Covering a broad objective, they have sought to determine possible relationships with bacterial infection, psychosomatic factors, abnormal body metabolism, iron deficiency anemia, and hormonal imbalance. A recent finding of significance is the presence of a transitional L-form of an alpha streptococcus, not only in the oral tissues of several patients suffering with the disease but in the blood stream as well. This organism was consistently recovered from lesions in numerous patients on repeated examination over a 12-month period. Both human and animal studies have indicated that hypersensitivity to the antigens of this organism is an important factor in the development of the lesions. Currently, attempts are under way to develop a specific antiserum.

This promising direction of study, being performed in collaboration with the Division of Biologics Standards, may provide additional leads as to the role of bacteria in other conditions such as desquamative stomatitis, erosive lichen planus, and various nonspecific stomatitis. Therapeutic investigation is also underway on all these stubborn and resistant chronic debilitating diseases, with limited success attributed to administration of various forms of achromycin and topical steroids.

In other clinical activities, emphasis has been given to the patterns of mucosal tissue change attributable to age; and to the elucidation of a previously undescribed "focal epithelial hyperplasia" in Indian children. In the latter study, field work is important to make available fresh material for viral cultures. Parallel studies are providing a description of histopathological changes associated with systemic disease, including amyloidosis and multiple myeloma, and an evaluation of mucosal changes following systemic chemotherapy. Studies of amyloid deposits, isolated from human tissue, carried on near the limit of resolution of the electron microscope, have revealed two types of particles. This marks the first step in developing an understanding of the structure of this material, which appears amorphous under the light microscope, and provides a base for future experiments by means of tis-

sue culture, biochemical, and X-ray diffraction methods.

In other oral pathology activities, a study of third molar follicles and follicular cysts has demonstrated that in teeth removed from patients over 26 years old, no enamel organ epithelium is found, i.e., the transition to epithelium of the squamous type is complete. These observations show that follicles, as such, do not develop keratinized linings despite increasing age, but that follicles which develop into cysts may do so. It was thus hypothesized that a decrease in the prevalence of ameloblastomas associated with follicular cysts after two decades may be explained on the basis of the transition from enamel organ epithelium to squamous epithelium.

Another clinically oriented study has dealt with the histopathology of tumors appearing in the oral, dental and related tissues of mice inoculated at birth with polyoma virus. These tumors apparently arise from gingival epithelium, nasal mucosa, accessory salivary glands and osseous tissue. Those arising within the incisal and molar areas presented several characteristics of human ameloblastomas.

In studies of the genetic control of cellular metabolism and chromosomes, *in vitro*, the effect of S₁₀ virus on transforming hamster cell lines into malignant cell lines has indicated that there is a chromosome instability which has continued for many generations. The latter phase of this investigation has been pursued in collaboration with the NIAID. In the chromosome study (conducted in collaboration with the NCI), it was found that an unusual hamster tumor (lymphosarcoma) has a particular stability for number of chromosomes; that is, the tumor cell may be identified by its chromosome number. By using this technique, it has been shown that the tumor may be transmitted from one animal to another animal either by injection of live tumor cells or by the transmission of the original tumor cells from one animal to another by the bite of a mosquito. Such findings may foreshadow the possibility that geographically localized tumors, such as African lymphoma, may be transmitted by an arthropod or insect vector.

Development of the Institute's oral exfoliative cytology program to include diseases other than cancer has received considerable attention in the past year. Rapid smear technics now in use have assisted importantly in the diagnosis of such non-neoplastic oral lesions as pemphigus, hereditary benign intraepithelial dyskeratosis, white sponge nevus, epidermolysis bullosa, Darier's disease, herpes simplex, and PPLO infections of the periadenitis mucosa necrotica recurrens type. Demonstrating characteristic and specific cell lesions, these technics are valuable for screening population groups, assisting in diagnosis when surgical biopsy material cannot be obtained, and in follow-up examination.

In another clinical field, a collaborative study with the Anesthesiology Department of the Clinical Center on general anesthesia in ambulatory dental patients is developing important information concerning the physiological effects of various anesthetic agents and oral surgical procedures. Since in some geographic areas, as many general anesthetics are administered in dental offices as in the local hospitals, and since there are no other such studies being conducted, the basic physiological data being assembled from this study should prove important for the specialty of oral surgery and the dental profession in general. Among the more significant findings assembled, to date, are: (1) a consistent hypertension in all ambulatory anesthesias, which directly parallels the intensity of the surgical stimulation; (2) the existence of preoperative and operative tachycardias in almost 100 percent of the anesthesias administered (the preoperative changes in rate are apprehensive in nature whereas the operative changes are due primarily to the pharmacologic action of the intravenous barbiturates and secondarily to surgical stimulation in the light anesthetic planes); (3) the demonstration that depression of arterial oxygen saturation is a controllable factor related to anesthetic management and drug administration (i.e., avoidance of obstructions and drug overdosage); and (4) the evidence that hepatotoxicity of Fluothane is extremely low.

In a collaborative study with NIAMD of Fraction I of Cohn, a fibrinogen and AHG

(anti-hemophilic globulin) concentrate, the Dental Institute's role has been to assess the response of hemophiliac patients to multiple dental extractions. The fraction, being a concentrate, allows the AHG adjustment in plasma without overloading the systemic circulation with plasma fluid. Results, to date, are most encouraging.

Prosthetic devices, although very often complicated and time-consuming to produce, frequently are necessary in the post-surgical management of intra- and extraoral head and neck cancer. Because of the severe emotional, nutritional, hygienic, and other changes associated with loss of oral and masticatory structures, the development and use of intraoral splinting techniques has been an important factor in total patient recovery or rehabilitation. During the past year an increasing number of maxillofacial devices have been constructed for patients with cancer, and an efficient working relationship developed between the dental and NCI surgery staffs. In one example of collaboration, the Dental Services Branch designs and fabricates a temporary obturator prior to maxillofacial surgery and makes insertion while the patient is still on the operating table. Such an appliance not only retains the surgical packing but also protects the surgical site, thereby allowing rapid return to more normal functions of mastication, swallow, and speech. Another temporary obturator is then constructed three weeks post-operatively, and retained until the surgical site is completely healed. At that time a permanent obturator is completed and maintained.

Another example of cooperative study and service is to be found in the post-surgical handling of laryngectomized patients where considerable difficulty had been experienced with

the conventional sterling silver tracheal tube. In general, patients using the stock tracheal tube complained of chronic irritation not only within the trachea but also in the skin area surrounding the stoma. Unhygienic conditions, reduced humidity, and the possibility of foreign objects entering the trachea presented additional drawbacks and difficulty in construction of a suitable prosthesis. Through appropriate modifications, a group of 15 laryngectomized patients are now wearing improved tracheal prostheses. These are one-piece, case-hardened pyrex glass appliances which are esthetic, hygienic, non-irritating and capable of maintaining humidity while protecting the trachea from debris.

A particular debt of gratitude is owed by the Dental Institute to its Board of Scientific Counselors. As in previous years, the Board's dedicated interest, enthusiastic support, and wise counsel provided further incentives for achievement and encouraged profitable exchange of ideas with particular relevance to long-range program planning.

It is apparent from the foregoing account of research activities that our professional attainments of today are a clear consequence of the extension of the boundaries of knowledge that have come about coincident with the intimate bonding of the health professions and related science disciplines within the total NIH complex of biomedical science. Obtainable for the professional family resident in this unique environment have been the highest degree of scholarship, the most complete understanding of the fundamental health sciences, the greatest range of choice of design, method and direction of research ventures, and the broadest freedom of their application; all existing under the least demanding of outside pressures.

DIVISION OF BIOLOGICS STANDARDS

INTRODUCTION

The Division of Biologics Standards, which has the responsibility for administering the provisions of the Public Health Service Act with respect to the control of biological products, discharges this task with activities about equally divided between control and research. The research programs are concerned largely with the function of the Division—the control of biological products—although by their very nature, some of these activities could well be classified as basic or fundamental research. However, no programs are undertaken initially unless they have direct bearing on the responsibilities of the Division. The research activities of the Division are therefore product-oriented, and their scope, direction, and intensity are dictated by the need to provide essential information for developing requirements and regulations for the licensing and release of biological products.

It is characteristic that the Division, with its ultimate objective the protection of the public against unsafe and ineffective biological products, is confronted with problems which are never completely solvable. In some instances difficulties can be anticipated, headed off, or abated. To accomplish this, however, it is necessary to maintain an active interest in products which have long since been routinely accepted by the medical and health professions, and in some instances even forgotten by the majority of scientists working within the field. The Division can never completely abandon a concern for any licensed product. Smallpox vaccine is an excellent example of this.

During the period covered by the present report, the most notable events were perhaps those which affected the staff of the Division. The formalization of certain organizational changes directed toward better functioning of the Division took place in October 1964. Dr. John D. Wagner, of the Director's staff, was

appointed Assistant Director. The Section on Pathology, formerly under the Laboratory of Viral Immunology, because the Laboratory of Pathology under Dr. Ruth L. Kirschstein, Dr. Harry M. Meyer, Jr., Acting Chief of the Laboratory of Viral Immunology (LVI), was appointed Chief of LVI, and Dr. John N. Ashworth, Acting Chief of the Laboratory of Blood and Blood Products (LBBP), was appointed Chief of LBBP.

The Division now consists of the following seven laboratories:

Laboratory of Bacterial Products -----	(LBP)
Laboratory of Biophysics and Biochemistry -----	(LBB)
Laboratory of Blood and Blood Products -----	(LBBP)
Laboratory of Control Activities -----	(LCA)
Laboratory of Pathology -----	(LP)
Laboratory of Viral Immunology --	(LVI)
Laboratory of Virology and Rickettsiology -----	(LVR)

The following staff members were appointed to the posts of section chiefs: Dr. John C. Feeley, Chief of the Section on Bacterial Vaccines (LBP); Dr. Edward H. Mealey, Chief of the Section of Blood and Blood Derivatives (LBBP); and Robert Pennington, Chief of the Section on Diagnostic Reagents (LBBP). With these changes, the Division is better able to carry out its dual function of control and research.

Two visiting scientists, Dr. H. Montes de Oca from Argentina and Dr. A. C. Hekker from the Netherlands; and two guest workers, Dr. Noboyuki Uchida from Japan and Dr. K. H. Dave from India made significant contributions to the Division's program during this period.

A number of technical problems were of major concern to the Division during fiscal 1965. Among these were the following:

During the late fall of 1964 it became apparent that the strains of A₂ influenza virus responsible for outbreaks of influenza in different parts of the world represented an antigenic shift from the A₂ strain (Jap. 170) represented in the current vaccine. Consultations were held with influenza experts and representatives of licensed manufacturers. After consideration of all the information available and after study of the antigenicity of trial vaccines prepared for a possible substitute A₂ strain, a recommendation for A₂ strain change was made. The A₂ influenza strain virus representation in the vaccine was divided equally between Jap. 170 and Taiwan/1/64 strains.

Another problem with respiratory virus vaccines presented itself when information from a number of sources, including members of the Division staff, indicated that adenovirus types 7 and 3 were oncogenic for hamsters. A conference was held on December 7 and 8 to review this information. Since there was uncertainty as to the significance of the finding with respect to the safety of adenovirus vaccines for human use, and since there did not appear to be any immediate solution to the problem, the Division advised manufacturers that no further lots of adenovirus vaccine would be released until the matter was resolved.

During this year, additional manufacturers were licensed for measles vaccine, so that by the end of June there were two establishments licensed for inactivated measles vaccine and five for live measles vaccine. Thus measles vaccine is readily available in amounts adequate to immunize all susceptible persons in this country.

Regulations governing clinical investigations with new drugs became effective during the early part of 1963. It is now necessary for those engaged in clinical investigation of biological products, to comply with the provisions of §130.3 of the Food and Drug Regulations and submit "Notices of Claimed Investigational Exemption for a New Drug" (IND's) to the Division of Biologics Standards. To meet the

demands of these new requirements, a separate activity continues to function within the Office of the Director of DBS, headed by Dr. Helen L. Tepper. Because regulations of standards for safety, purity, and potency must be in existence before a biological product can be licensed, the Division must keep in close contact with those who are carrying out field and other investigations concerning the safety and potency of products so that the formulation of regulations or standards can be accomplished against a sound background of knowledge and experience. During the period covered by this report 46 IND's and 212 supplements were submitted to this Division.

Investigation of alleged violations of the "Biologics Law" continues to be demanding of the time of Division personnel. Attention to detail in the collection of evidence which will stand up in court requires that those involved devote most of their time to this activity. Accordingly, an investigations group has been organized within the Office of the Director. Judgments handed down in connection with these cases should have a salutary effect on any operators who wish to short-cut the safety and potency requirements of the Federal statute and regulations. However, the number of cases under current investigation would seem to indicate that the message has not been widely received. Litigation in connection with alleged cases of poliomyelitis, occurring in association with administration of live polio vaccine has continued, and members of the Division staff have been called in a number of such cases.

Construction of additional facilities for the Division commenced in March 1965, and it is anticipated that building 29A will be completed by December, 1966. This will provide much needed laboratory space and enable the Division to conduct a more evenly balanced program of research and control.

The following summaries of the programs of each of the laboratories present in more detail the activities of the Division.

LABORATORY OF BACTERIAL PRODUCTS

With the recent reorganization in the Office of the Director, a greater responsibility has

been placed on the Laboratory of Bacterial Products relating to the review of license applications and labeling for allergenic and bacterial products, and in the performance of other non-research functions. The number of applications for licenses for allergenic products has been much above normal. These duties have been carried out without an increase in staff. We look forward to an expansion when additional space becomes available within the next two years. During the year the professional staff has remained the same except for an exchange of personnel in one position. One member has been awarded a Ph. D.; her thesis was based on an assigned research project.

In addition to the current projects, it is considered that emphasis should be placed on the development of U.S. Standards of Potency for allergenic extracts (at present there are none) and reevaluation of standards for BCG and plague vaccines and staphylococcus products. Additional work is needed on the potency test for typhoid vaccine to eliminate possible false high potency values influenced by high Vi content. Emphasis also should be placed on the preparation of Additional Standards for all allergenic and bacterial products for incorporation in Public Health Service Regulations.

Standards

During the year progress was made towards the development of specifications for several products. Studies carried out in cooperation with the Laboratory of Control Activities were directed towards coccidiocin, toxoplasmin, the evaluation of the new reference preparation for typhoid vaccine, statistical analyses of the tuberculin potency test, and participation in the titration of the potency of a new international reference preparation for Tuberculin, Old and an international reference preparation for Tetanus Toxoid, Adsorbed. Recommendations relating to the Manufacture of Cholera Vaccine were prepared and issued preliminary to preparation of Additional Standards: Cholera Vaccine. Potency standards for Pertussis Immune Serum Globulin (Human) were drafted.

Research Projects

Cholera Vaccine and Cholera Vibrios The continued prevalence and spread of cholera outside of the age-old Bengal endemic area continues to stimulate research on cholera. Special emphasis has been placed on laboratory potency evaluation of vaccines in relation to human efficacy, classification of the cholera vibrios and molecular size of antibodies that afford passive protection in the infant rabbit cholera model. Vaccines used in the field trials in East Pakistan, Calcutta and the Philippines have been or are being assayed by the DBS mouse protection test which in WHO cooperative studies has been shown to be better than the Sokhey-Habbu mouse test. A highly potent vaccine used in East Pakistan has given significant protection against Inaba serotype infection for at least 18 months whereas in Calcutta and the Philippines less potent vaccines have given low protection aid been of short duration. However, the infections in the latter places have been caused by Ogawa serotype vibrios. There is a suggestion that protective antigens of the different serotypes may be quite different in activity.

A proposed classification of *Vibrio cholerae* based on subtype characteristics has been favorably received and if adopted would eliminate the confusion that has arisen by attempts to designate the so-called "El Tor" vibrios as a separate species. Passive protective activity of anticholera serum for infant rabbits has been shown to be related to 7 S antibodies and not to agglutinin and bactericidal titers.

The Chief, LBP, has continued to serve as the NIH Project Officer for the Pakistan-SEATO Cholera Research Laboratory and as a member of several advisory committees.

Pertussis Vaccine

Two pertussis vaccines have been especially prepared as candidates for a toxicity reference vaccine. Emphasis will be placed on correlating the laboratory assayed toxicity with reactivity of vaccines in children. The studies will be correlated with similar ones in Great Britain and Japan. Studies on effect of preservatives on stability of potency as well as toxicity will be

continued. Merthiolate remains the best preservative.

Tetanus Toxoids

In the collaborative study on the prevention of neonatal tetanus it has been shown that an antitoxin titer of not more than 0.01 u/ml is adequate to prevent neonatal tetanus, and that protective titers following injections of AlPO_4 adsorbed toxoids persist for at least two years. Although a single dose of one oil adjuvant toxoid induced good antitoxin response and with a low reaction rate, subsequent preparations induced such severe reactions that further use of oil adjuvants with tetanus toxoid is contraindicated. Studies so far have failed to pinpoint the cause. In the overall study the duration of immunity and the response to booster inoculation is being followed.

From the cooperative WHO study on a reference adsorbed tetanus toxoid, a unit of potency can be assigned to the absorbed toxins used in the field trial. This information will be valuable in assigning specific potency requirements for tetanus toxoids.

Studies on the separation of antigenic components of culture filtrates of *Clostridium tetani* have yielded a high degree of separation of neurotoxin and hemolysin. Other constituents although difficult to separate and analyze are showing interesting activities which may be related to untoward reactivity.

Pleuropneumonia-like Organisms

In collaboration with members of the Staff of the National Institute of Dental Research it has shown a high incidence of transitional L-forms in recurrent aphthae lesions. In collaboration with the Clinical Center and the National Cancer Institute the role of mycoplasma in diseases of man is being investigated. Certain mycoplasma showed agglutination in anti-I serum and this mimicked the antigen I negative finding in leukemia patients. Mycoplasma has been isolated by direct culture procedures from bone marrow of leukemic patients. By electron microscopy particles indistinguishable

from mycoplasma have been seen in tissues taken from these patients.

Tuberculin

In the continuation of their studies on the characterization of components of unheated culture filtrates of BCG, Doctors Baer and Chaparas have shown conclusively that a carbohydrate component induces delayed skin reactivity in sensitized guinea pigs to the same degree as protein fractions. The delayed-skin reactivity was the same in animals with or without detectible serum antibody to the carbohydrate. With the use of *Nocardia asteroides* in place of mycobacterium in Freund's adjuvant it has been possible to sensitize guinea pigs to isolated fractions of tuberculin filtrates. This tool should be very helpful in determining if tuberculins from different mycobacteria contain reactive substances that would be diagnostically specific.

Poison Ivy

Methods of extraction, isolation and identification of the active principles of poison ivy are being investigated. The activity of the agents isolated are being studied using guinea pigs. Clinical studies are being made to correlate human and skin guinea pig reactivity and to determine if poison-ivy-sensitive humans can be desensitized. The development of a standard of potency for poison ivy is being much more complicated than previously thought.

LABORATORY OF BIOPHYSICS AND BIOCHEMISTRY

Research Program

Microbiol Biophysics

Investigations of the inactivation of animal and bacterial viruses continue to represent the major research accomplishment of the laboratory, although this phase of the program has been reduced in emphasis to allow expansion and solidification of other laboratory activities. An interesting study of phenotypic mixing between adenovirus 4 and SV40, as revealed by thermal inactivation data has been completed.

Analytical Chemistry

Development of methods and equipment for analyzing biological products and training of personnel have absorbed most of the efforts of the chemical analysis group. Installation and modification of automated equipment for nitrogen analysis and an automatic absorption spectrophotometer for analysis of metallic elements have been completed. The analytical ultracentrifuge and associated equipment has been transferred to this group where it will be operated primarily in a service capacity.

Electron Microscopy

Operations of the electron microscopy unit have continued at the level of a service function pending recruitment of a scientist of demonstrated accomplishments in the field and with a sustained interest in virus morphology. According to present plans, this position will be filled in September 1965, with a consequent expansion and intensification of the program.

Control Activities

During the calendar year 1964, chemical analyses were performed on 97 samples of biological products representing the products of 19 licensed manufacturers, of which 13 were domestic commercial establishments, 4 were State laboratories, and 2 were foreign manufacturers. Nineteen different types of products were analyzed by one or more of the 14 different analyses which are now available within the repertoire of the group. The data collected in these analyses form the nucleus of a continuing collection of base-line or normal analytical values for biologicals.

Prelicensing examination of collagenase as a potential biological product called upon the facilities of the laboratory to test and verify the manufacturers method for measuring potency. The analytical program also illustrated its relevance to the DBS control function when an inspection sample of a licensed product was found to contain substantial amounts of pyridine. Subsequent investigation disclosed a faculty manufacturing practice which permitted inadvertent contamination of the product with this substance.

LABORATORY OF BLOOD AND BLOOD PRODUCTS

Intramural Research Activities

The research program of the Laboratory of Blood and Blood Products has included studies of the stability of blood products, the development of methods and standards for these products, the investigation of red cell antigens, plasma antibodies, and the clotting and fibrinolytic systems, and the examination of proteins of body fluids. These various fields of interest have as their goal the improvement of procedures used for the control of the purity, potency and safety of biological products derived from blood. Thus the projects are directed toward improving existing control tests, developing new ones, obtaining stability data leading to more realistic dating periods, and providing the professional staff with constantly updated information necessary in evaluating new products and procedures.

Investigation of the stability of human albumin during long-term storage is typical of the Laboratory's program of applied research. These studies have shown that under proper storage conditions albumin can be relatively stable for a period of 10 years. Stability depends on the storage temperature, protein concentration and the source from which the albumin is prepared.

Basic research done in the Laboratory has dealt with various phases of the clotting and fibrinolytic processes. In the course of these studies a method has been developed for the isolation, in good yield, of a preparation of antihemophilic factor with specific activity 50 to 60 times greater than that of fresh plasma.

Control Activities

The primary function of the Laboratory is the control of the purity, potency and safety of blood and blood products. Approximately three-fourths of the total time of the Laboratory personnel is occupied by this function. Control activities included the inspection of 173 licensed establishments at a total of 286 locations and the investigation of 8 possible vio-

lations of the Public Health Service Act. This required 466 man days of travel. Laboratory personnel performed a total of 42,427 tests on 2743 samples of blood products submitted for release, licensure, or as a result of inspections. License applications for 70 products and 14 establishments were reviewed and forwarded for approval. More than 450 groups of labels and circulars for blood products were reviewed for compliance with regulations. In addition, the development of Technical Standards for biological products derived from blood constituted an integral part of the Laboratory's control activities.

Extramural Research and Training Programs

The extramural program of the Laboratory is not formally organized in the sense of extramural programs of the other Institutes. However, the nature of the control functions provides opportunities for numerous contacts with scientific staffs of outside organizations. An example of this type of activity was the Fourth Voluntary Blood Bank Evaluation Study. Results from 169 of the licensed establishments participating in this study were received and tabulated by Laboratory personnel. This required the classification of nearly 14,000 tests results in order to compare them with those of the four reference laboratories. A copy of the tabulated results and a brief summary of the major findings were sent to each participating establishment.

To supplement the research activities of the Laboratory and yield specific information necessary for the development of regulatory standards, five research contracts are in force. The project titles of these contracts are:

- (1) Hemagglutinin Levels of Normal Human Plasma
- (2)
 - a. Optimal Standards of Potency for ABO Blood Grouping Serums
 - b. Development of Standards for Anti-Human Serum to Detect ABO Sensitization
- (3) Anti-Human Protein Serums for Specific Identification of Protein by Diffusion Methods
- (4) Reagent Red Blood Cell Reference Panel

(5) Laboratory Studies of the Purity of Plasma and Plasma Fractions Stored in Plastic Containers

In support of its control functions the Laboratory carries on an inspector training program. This program is designed for the training of new inspectors and to provide the opportunity for experienced inspectors to discuss interpretations of regulations and problems encountered during inspections.

LABORATORY OF CONTROL ACTIVITIES

The Laboratory of Control Activities has prime responsibility within the Division for performing and coordinating the testing conducted on biological products to determine their conformance to standards of safety, purity, and potency. The laboratory is composed of three sections i.e., Control Testing, Pyrogens and Reference Standards, and an administrative technical staff which performs the voluminous task of reviewing manufacturers' product protocols, and coordinating, compiling and filing a major portion of the data of manufacturer submitted to the Division in connection with these control activities.

Control Activities

The Laboratory has as its primary responsibilities the following activities:

(a) To determine whether each lot of a licensed biological product meets prescribed standards of safety, purity and potency and to exercise the necessary control actions culminating in either official release or rejection of the material intended for sale by the manufacturer in interstate commerce. Such action is based on the results of a detailed review of the manufacturers' processing and testing data as reported in manufacturing protocols, testing data compiled within the Division, and any other available source of information relating to the safety, purity or potency of the product.

(b) To develop, maintain and distribute physical biological standards, references, control materials and reagents necessary for the testing of products to determine compliance with standards. A culture collection or deposit is also maintained to support the technical

effort of the Division as well as to support the control activities as they affect the licensed manufacturers.

(c) To maintain under review published standards, minimum requirements, technical memoranda and other guidance issued by the Division to manufacturers as concerns licensable biological products and to develop or propose revisions in regulations when indicated, and to prepare technical specifications and other regulatory procedures for new products as they are developed.

(d) To maintain a close liaison and working relationship with other laboratories of the Division and where indicated of the Institutes as well as other outside agencies to insure a continuous flow of knowledge and technical information deemed vital for the proper exercise of control measures of both those products already licensed and new products being developed for license.

(e) To participate in and perform annual inspections of designated licensed establishments in accordance with specified procedures and for specific objectives as prescribed by law.

(f) To participate in and give technical support to investigations performed by the Division in connection with Public Health Service Act.

(g) To participate in the review of applications and reports of manufacturers and their products to determine eligibility for license.

The scope of the activities carried out by the Laboratory of Control is shown by the fact that, during the past twelve-month period (January 1, 1964 to December 31, 1964), a total of 6,375 control tests were performed by the laboratory staff to insure that lots of licensed biological products met prescribed standards of safety, purity and potency. Of this number, 5,599 tests were conducted on products for release, 760 tests were made on inspection samples and 16 tests were performed in connection with complaint investigations. The results of these tests in a majority of instances, served as a basis for the Division taking either release or rejection actions or recall by the manufacturer of the product lot depending upon the situations. In addition 1747 cooperative service and standardization tests were performed on biological products not licensed but

in which the Division and/or the Public Health Service had a direct interest.

During the past calendar year, 3,980 lots of licensed biological products together with their manufacturing and testing protocols were submitted by manufacturers to the Division for consideration. Of those 3,884 lots were released, 52 lots were rejected and 44 lots were withdrawn from consideration by the manufacturers.

The reference standards section of the laboratory maintains a current inventory of 80 official standard reference and control preparations for use by the Division, by licensed manufacturers and by other qualified investigators in connection with the standardization and control testing of biological products. An adequate supply of these materials must be available at all times, and in support of this capability 13 antitoxins, 5 antiserums, 4 vaccines and 1 toxin material were prepared and standardized during the year. A total of 354 tests were required to complete a satisfactory standardization of these materials. Standards, reference preparations and cultures are, whenever possible, freeze dried and sealed under vacuum or dry nitrogen in order to maximize the probability of maintaining stability during storage and distribution. During the past calendar year the following classes and amounts of these materials were dried by the reference standards section:

	<i>Ampules</i>
Viable cultures:	2558
Serums -----	63
Vaccines -----	3280
Viruses -----	1541
Toxins -----	178
	7620

Standards, reference preparations and cultures were distributed on request to research and control laboratories of both licensed and potentially licensable manufacturers, state and federal health laboratories and qualified university research groups. The amounts distributed were as follows:

	<i>Ampules</i>
Antitoxins -----	381
Serums -----	1863
Vaccines -----	2394
Bacterial and Viral Cultures -----	1301
Total -----	6191

Research Activities

SNAKE ANTIVENIN. Research related to the development of standards for the preparation and potency testing of coral snake antivenin have been initiated during the year. The studies will extend to the investigation of the properties of coral snake venom and antivenin concerned with protecting man from the coral snake bite.

TOXOPLASMIN. Studies on the development of an *in vivo* laboratory test procedure for standardizing the potency of toxoplasmin employed in skin testing are underway and should provide a basis for the eventual drafting of potency standards for the product.

Coccidioidin. Studies to develop a suitable laboratory procedure and reference coccidioidin preparation for use in the potency testing of the product are underway. Such a testing procedure and reference preparation will have application in the regulation, control, and standardization of the licensed product.

Other research studies relating to the development of an improved method for potency testing and standardization of Diphtheria Toxin for the Schick Test, Smallpox Vaccine, Tetanus Toxoid, Tuberculin, and Typhoid Vaccine were carried out during the year and are in various stages of completion. Interim reports have been made summarizing the findings with the ultimate expectation that such information may be used as a basis for the formulation of more definitive standards.

LABORATORY OF PATHOLOGY

The Laboratory of Pathology was created during 1964 and consists of two sections, a section of Animal Testing and a Section of Histopathology. These two sections are responsible for the testing and research activities involving a variety of viral vaccines. In addition, the Section of Animal Testing collaborates with other laboratories in the Division in research projects involving the inoculations, observations and removal of tissues from monkeys. The Section of Histopathology, besides preparing the tissues and processing the histologic sections for the monkey neurovirulence tests

for poliovirus vaccines, measles vaccine and yellow fever vaccine, processes tissue for research programs performed in collaboration with other laboratories in the Division. The Histopathology Section of the Laboratory of Pathology cut and stained 27,485 histologic sections, 12,699 with galloxyanin and 12,780 with hematoxylin and eosin or special stains. 2600 blood smears and 500 cover slip preparations were prepared. This is approximately 556 slides per month per technician.

The Laboratory of Pathology will continue to have, as its main function, the performance of neurovirulence tests of viral vaccines in monkeys. However, the laboratory also collaborates in any testing of biological products which may require either special or routine histologic study such as studies leading to the qualification of measles seed strains for vaccine production.

During the year covered by this report the laboratory collaborated with members of the staffs of the Laboratory of Bacterial Products, the Laboratory of Viral Immunology and the Laboratory of Virology and Rickettsiology in research problems involving the pathogenesis of viral diseases such as rubella and infectious hepatitis, and the pathogenesis of immune responses and the reaction of tissues to emulsified antigens.

In addition, the Laboratory has pursued its own research interests in oncogenic viruses and particularly in the relationship of oncogenesis to the immune response and in the pathogenesis of acute viral infections. The Laboratory is continuing its studies of neuroanatomical development of various animal species and its relationship to the pathogenesis of diseases induced by neurotropic agents.

The Laboratory now has on its staff a neuroanatomist who is well versed in histology, cytology and embryology and a veterinarian who has had a wide experience in the care of primates. It is planned to enlarge the experience of both these staff members so that they may become adept at interpretation of abnormal histologic findings.

It is fully expected that the activities of this laboratory will expand as collaborative proj-

ects with many other staff members of the Division increase and as studies involving other biological products demand histologic examination.

LABORATORY OF VIRAL IMMUNOLOGY

During the period covered by this report the Laboratory of Viral Immunology has conducted a biologics-related virus research program which included studies in both basic and applied virology. The highlights of these activities are summarized in the paragraphs that follow.

Rubella

The isolation of the virus three years ago, coupled with the major rubella epidemic of the past year, have focused a great deal of attention on rubella research. In LVI, this work has been along three general lines:

- (1) Search for a useful experimental animal;
- (2) Development of serologic procedures and
- (3) Basic studies of the biological properties of the virus.

Creditable progress has been made in all three categories. The virologic events characterizing experimental infection induced in the rhesus monkey have been shown to resemble closely those occurring in human rubella; these animals experience viremia, shed virus in the respiratory and intestinal tracts and uniformly develop neutralizing antibodies.

There has been a real need for an animal model, especially for use in investigation that would be difficult or impossible in man. Exploiting the research potential of the simian model, evidence has been obtained that inoculation of rhesus early in gestation results in an infection that is transmitted to the products of conception. Also, monkeys have been used to evaluate rubella virus neurovirulence and to conduct an *in vivo* therapeutic trial of the anti-viral drug, L-adamantanamine HCl.

In other laboratory studies, an improved neutralization test was developed and a number of important basic biological charac-

teristics of the virus delineated. These investigations are described in detail in the individual project descriptions.

Measles

The past year has seen an increased national effort to control measles. Four new live measles virus vaccines were licensed. One measure of the effectiveness of the Division's measles program has been the smoothness with which these new products were evaluated, licensed and released for clinical use. Laboratory studies by the Division, followed by individual conferences with manufacturers, detected and resolved several problems well before licensure.

Maintaining an active interest in the complexities of measles control in developing nations, the LVI has collected epidemiological data from the Republic of Upper Volta that should prove useful in guiding future field use of the vaccine. The immunization of 731,000 children in the DBS-directed mass campaign of 1962-63 has significantly reduced the incidence of epidemic measles in Upper Volta during the past two years.

In the laboratory a new hemagglutination-inhibition antibody test using automatic mechanical equipment has been developed and standardized. This technique provides a greatly increased potential to the LVI for assaying the measles antibody content of human gamma globulin and sera from children participating in measles vaccine trials.

Other facets of the measles research program are described in the project reports.

Poliomyelitis

An exciting development of the past year has concerned an immunochemical approach to the evaluation of poliovirus virulence. Using a quantitative complement-fixation technique it was possible to differentiate between virulent and attenuated poliovirus strains of the same serologic type. This method offers a new *in vitro* approach to differentiating viruses immunologically indistinguishable by conventional techniques.

The results of serologic studies using the metabolic inhibition neutralization test have

suggested a shift in the antibody pattern in the adult population, for poliovirus. Gamma globulin prepared from recently collected plasma pools usually contains neutralizing antibodies in higher titer than comparable material processed in past years. This change is thought to be a result of the widespread use of poliovirus vaccines.

Smallpox

Smallpox vaccine is the oldest of the biologic products, yet epidemics of the disease continue to occur in much of the world. The World Health Organization and others have made world-wide smallpox eradication a goal. The ferment in this field is leading to a re-evaluation of old vaccination practices and a search for new techniques and vaccines. The pioneering work of the Division in jet inoculation of smallpox vaccine and mixtures of smallpox and other vaccines has been in anticipation of the new philosophy. The LVI is continuing its investigations of the nature of the clinical reaction and immunologic response of man and experimental animals to jet-inoculation of vaccinia virus.

Mumps

Epidemic viral parotitis is another of the common diseases of childhood that may in future years be controlled by vaccination. An inactivated virus vaccine available for a number of years has had little clinical use since the protection resulting from inoculation has been marginal and transient. Russian experience with live, attenuated mumps vaccines has stimulated a certain amount of interest in the United States. Anticipating possible activity in this area, the LVI has initiated a new program that to date, has concentrated on characterizing the performance of several mumps virus strains in cell cultures and in developing sensitive methods for antibody assay.

Virus-Induced Neoplasms

Determining the possible role of viruses in the neoplastic process is one of the most important basic problems facing the biological

sciences today. One promising approach has concerned study of virus-tumor-cell relationships. The Section on Viral Genetics of LVI has used SV₄₀ and *in vitro* cultures of sarcomatous cells induced by this virus, as an experimental model. These studies have investigated some of the factors which can cause the malignant cell to convert the virus genome into mature, infectious SV₄₀.

In another project dealing with viral oncogenicity, the Section on Serology, in collaboration with the Laboratory of Pathology, obtained evidence indicating that one of the simian adenoviruses (SV₁₁) induces malignancies in hamsters. Such observations extend our knowledge of the spectrum of viruses with an oncogenic potential.

A New Adventitious Agent of Cell Cultures

In recent months a microsporidial protozoan, tentatively classified as *Nosema cuniculi*, was recovered from "normal" primary rat kidney cell cultures. Review of the literature suggests that this agent is a common pathogen of dogs, rodents and perhaps other animal groups. There has been limited evidence incriminating it as a central nervous system pathogen of man. The Section on Serology has found that the protozoan propagates well in tissue cultures of human, canine and rodent origin. These studies are continuing to determine if the agent is a significant potential hazard in tissue culture-produced vaccine.

Cell Culture Studies of Inherited Metabolic Disorders of Man

A continuation of this project conducted in collaboration with the National Heart Institute showed that the defect in phospholipid metabolism of Niemann-Pick disease could be demonstrated in diploid cell cultures prepared from tissues obtained from patients, but disappeared after the cells became heteroploid in serial propagation. In addition, biochemical analysis indicated that the genetic defect probably is in catabolic activity rather than in synthesis.

These groups are currently evaluating the possible usefulness of cell culture methods in the study of two other hereditary disorders of

metabolism; homocystinuria and Tangier Island disease.

LABORATORY OF VIROLOGY AND RICKETTSIOLOGY

The face of this Laboratory has continued to change during the past year. In November the inter-change of Sections between LVR and LVI was officially recognized. For the time being, however, the reality of the five Sections in operation since early 1964 has not been reflected in the Table of Organization. The officially listed sections still are: Respiratory Viruses (Dr. Morris), Biology of Viruses (Dr. Li), Tissue Culture (Dr. Shelokov—Acting) and Basic Virology and Rickettsiology (Dr. Shelokov). In practice the last Section has been divided into two: Rickettsiology (Mrs. Hopps—Acting) and Experimental Virology (Dr. Shelokov). In line with the planned addition of research competence to the traditional service functions of the Tissue Culture Section (with eventual re-designation as Cell Biology), H. Montes de Oca, M.D. was appointed as Visiting Scientist. The increasing concern of the Rickettsiology Section with problems of cell parasitism (rickettsiae, PPLO and large viruses) is responsible for addition of Richard Mason, DVM, Ph.D. (in Microbiology) with experience in host cell-parasite research. The newly created Section on Experimental Virology has concerned itself with arboviruses, avian leukosis complex and hepatitis viruses.

Respirovirus Studies

These concentrated on the problem of current antigenic shift in A₂ influenza viruses. The results prompted the Division to add A/2/Taiwan/1/64 to the influenza vaccine formula for the next season. The presence of antibodies to the influenza A₂ viruses was studied in human volunteers in relation to immunity to challenge with live A₂ virus, providing further evidence for the importance of homotypic antibodies in the natural and induce defense mechanisms.

Antimicrobial Agents in Foodstuffs

The emphasis has shifted from the effect of clam extracts on acute bacterial and viral in-

fections in animals and in cell cultures to their effect on virus-induced tumors, particularly by adenovirus type 12. A contract is under negotiation now to produce large amounts of clam extracts for further study here and elsewhere particularly in regard to their inhibition of viral oncogenesis. In the meantime, the Section is beginning to formulate other projects concerned with the biology of viruses.

Viral Hepatitis

A new approach to the study of A-1 virus has emphasized its experimental adaptation to an animal host. Serological studies by the plaque reduction technique are continuing. Promising new approaches to the broad problem of hepatitis virus candidates are being explored.

Rickettsiae

Interferon production by various rickettsiae in cell culture and laboratory animals was studied during the year. Other work concerned comparative electron microscopy of rickettsiae, larger viruses and PPLO and the effects of the presence of PPLO on the cellular response to viral infection. Search for an optimal immunization schedule with killed epidemic typhus vaccine is continuing; complement fixation response to typhus vaccines in the human subject was compared with standard guinea pig potency test as a means of vaccine assay. Increased interest in the use of attenuated rickettsial vaccines prompted an investigation into characterization of virulent and avirulent strains of certain rickettsiae of epidemic importance.

Arboviruses

This new project has produced a comprehensive arbovirus collection of not only seed viruses, but infectious pools, standardized antigens for complement fixation and hemagglutination-inhibition, and standardized antibody reagents. Studies on several specific arboviruses, including members of Bunyamwera and California groups important for the U. S. and the tropical viruses related to the sandfly host are well on the way.

At least under laboratory conditions, administration of naturally nonpathogenic Tacaribe virus has been shown to protect susceptible guinea pigs against the otherwise fatal infection with Junin virus of Argenian hemorrhagic fever; this may provide an important link in the development of an effective vaccine against the Bolivian hemorrhagic fever. An epizootic of an acute hemorrhagic fever among monkeys held at the Primate Quarantine Unit at NIH, has been studied in collaboration with Laboratory Aids Branch, DRS; the infectious and contagious nature of this disease in monkeys was established; adaptation of the virus to other laboratory animals and cell cultures is underway.

Avian Leukosis Virus

While RIF-testing of all appropriate vaccines is continuing, complement fixation (employing WRAIR-developed quantitative technique adapted here to microtiter equipment) was added as a regular procedure, and a fluorescent antibody facility has been set up to provide a comprehensive system of vaccine testing and monitoring of cell systems and of eggs.

Safety Testing of Inactivated Virus Vaccines in Cell Culture

Decreasing use of inactivated polio and measles vaccines and the problems with standard adenovirus vaccines were reflected in a smaller number of such products tested: only 11 lots of 3 inactivated vaccines were tested by Tissue Culture Section during the calendar year 1964.

Preparation of Cell Cultures for Research and Vaccine Test Use

The Tissue Culture Section continued to provide for the cell culture needs of the Division: During the calendar year 1964, 464,071 tubes, 111,045 2-ounce bottles, 2,229 milk dilution bottles and 3,327 32-ounce bottles of primary and serial cultures were prepared from a variety of standard and experimental cell sources.

APPENDIX

Scope of Control Testing Program

Since the individual reports taken separately do not reflect the magnitude of the control testing program which extends through each of the laboratories, a number of tables have been prepared to show the scale of the Division-wide effort. These data however represent only one phase of the control of biological products.

Essentially the control testing program operates in the following manner. Manufacturers of a product are requested to submit to the Division samples from each lot of the product manufactured along with a protocol containing the results from all tests performed on that lot. Some 290 different biological products are currently licensed. Samples and protocols are submitted on approximately 175 of these.

Each protocol and sample is reviewed by the Division to determine if the manufacturer's test results conform with established standards. In addition, some or all of the tests required on a particular product may be performed by the Division. If the manufacturer's test results and the results of tests performed by the Division conform with standards, a letter of release for that lot is sent to the manufacturer. Only then may the manufacturer distribute this particular lot for sale.

During the last year 3,884 lots of biologic products were released, 52 lots were rejected and 44 lots were withdrawn by the manufacturer. Table No. 1 reflects the number of lots released by categories of products.

A protocol may consist of up to 200 pages, for example, on a single lot of Poliovirus Vaccine, or only two pages for a lot of blood grouping serum. All protocols are recorded and reviewed by members of the Laboratory of Control Activities. The protocols are then forwarded to other laboratories within the Division for review. The nature of the product determines the laboratory consulted. Table No. 2 reflects the number of protocols reviewed by each laboratory. It does not however reflect the total number of people reviewing a particular protocol.

Although it has been indicated that the work load of the personnel of the Division is divided about equally between research and control activities, this does not reflect the number of people engaged in control functions. Some of the Division personnel are involved full time in the control program while many other individuals are involved on a much smaller scale. As the control testing on products becomes more

specialized, a larger number of the research scientists are becoming involved in control activities.

Table No. 3 reflects the total number of control tests performed by Division personnel last year. This gives no idea of the total effort involved however since tests requiring 15 minutes or less are given the same weight as those taking a month or more to complete.

January 1, 1964 through December 31, 1964

Table 1.—Number of lots of biological products considered in 1964.

Products	Lots released	Lots rejected	Lots withdrawn
Antitoxins-----	31	0	0
Therapeutic Serums-----	8	0	0
Blood and Blood Products-----	1665	5	4
Bacterial Vaccines-----	66	2	0
Toxoids and Toxins-----	49	0	0
Multiple Antigen Preparations-----	164	4	6
Viral and Rickettsial Vaccines-----	625	8	9
Diagnostic Substances for Dermal Tests-----	144	0	0
Diagnostic Substances for Laboratory Tests-----	993	32	25
Miscellaneous-----	139	1	0
Totals-----	3884	52	44

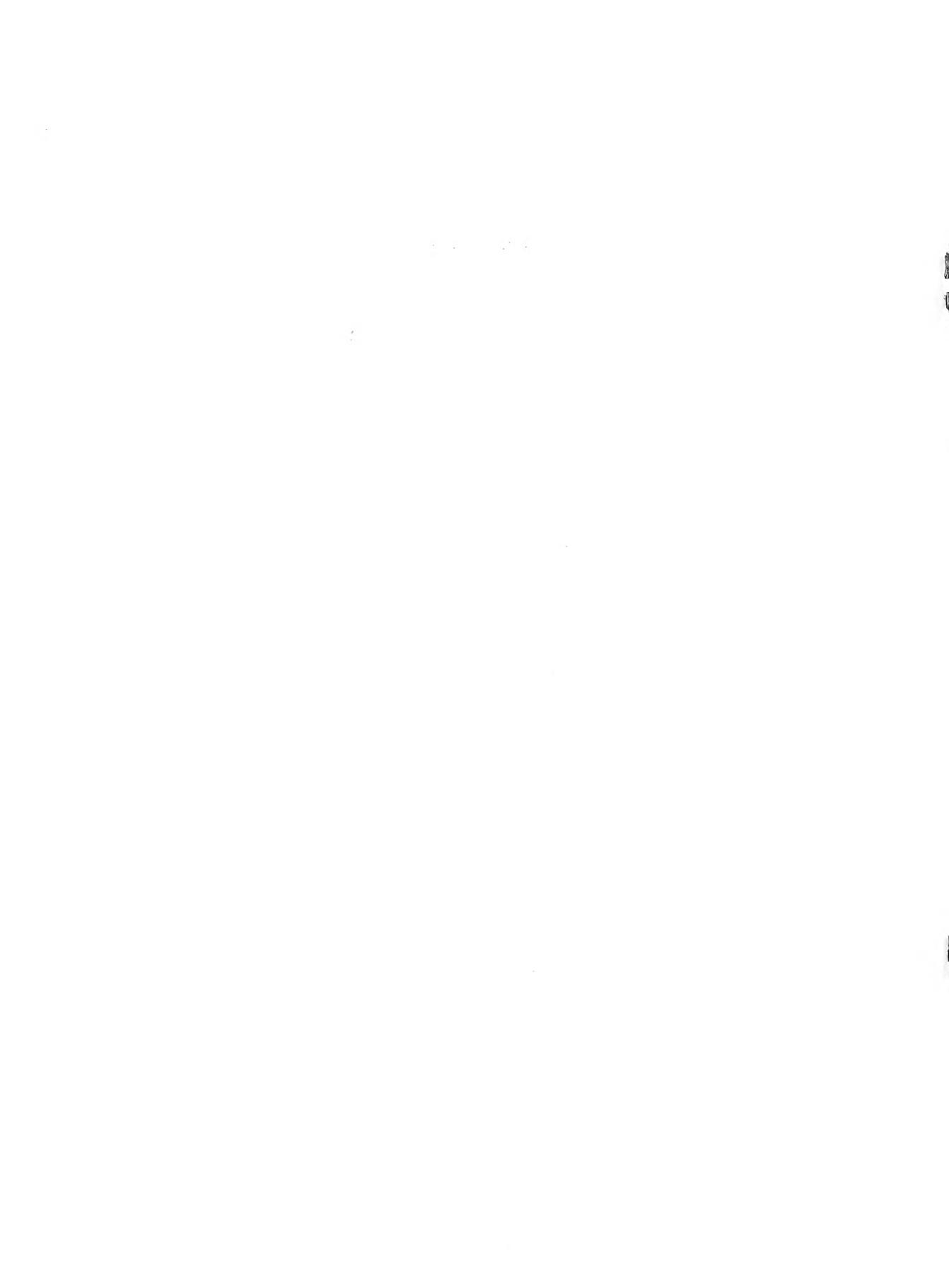
Table 2.—Number of protocols reviewed in 1964 by laboratory.

Products	LBEPI	LBP	LCA	LVI	LVR	LP
Antitoxins-----			31			
Blood and Blood Products-----	1674	3	1674	77		
Therapeutic Serums-----		3	8			
Bacterial Vaccines-----		9	68			
Toxoids and Toxins-----			49			
Multiple Antigen Preparations-----		88	174			
Viral and Rickettsial Vaccines-----		66	642	183	177	101
Diagnostic Substances for Laboratory Tests-----	1048	6	1051			
Diagnostic Substances for Dermal Tests-----			043			
Miscellaneous-----	134		140			
Totals-----	2856	175	3980	260	177	101

Table 3.—Total control tests performed in 1964.

Type of Test	Type of Sample Tested			
	Product for Release	Inspection	Complaints	Totals
Potency				
In vivo	1,858	45		1,903
In vitro	15,258	32		15,290
Safety				
General	755	24		779
Specific	325	8,988		9,313
Purity	20,704	7,531	9	28,244
Sterility	3,289	664	9	3,962
Total control tests				59,491

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